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**Characterization of human gene products homologous to
fission yeast multi-drug resistance determinants**

**A thesis submitted for the degree of
Doctor of Philosophy**

Discipline of Life Science

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**Institute of Molecular Medicine
Oxford
Sponsoring Establishment for
The Open University**

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ABSTRACT

Novel mechanisms involved in the resistance to cytotoxic agents have been recently described both in fission yeast and in mammalian cells. The work described in this thesis was focused on novel determinants of multi-drug resistance in mammalian cells and their possible mechanism of action.

Human Poh1, a highly conserved subunit of the regulatory particle of the 26 S proteasome complex, has been shown to confer moderate resistance to chemotherapeutic drugs and ultraviolet light in mammalian cells by a P-gp-independent mechanism. The mechanism by which HA-Poh1 causes drug resistance was investigated. Using constitutive and inducible expression of HA-tagged Poh1 in mammalian cells, it was found that most overexpressed Poh1 is not associated with the 26S proteasome complex. The cells expressing HA-Poh1 exhibited elevated protein levels of the two AP-1 components, c-Jun and c-Fos, associated with an increased stabilisation of c-Jun possibly caused by an interaction between HA-Poh1 and c-Jun. Furthermore, HA-Poh1 overexpression led to an increase in AP-1 transcriptional and DNA binding activities. As c-Jun and c-Fos are both degraded by the ubiquitin/proteasome pathway, it is proposed that Poh1 is able to negatively modulate the ubiquitin-dependent proteolysis of transcription factors with consequent alteration of cellular drug susceptibility.

PWPI is member of the WD-40 repeat protein family and is the closest known human relative of the fission yeast *pwp1*⁺ multi-drug resistance gene. It is shown here that overexpression of human Pwp1 in mammalian cells leads to multi-drug sensitivity possibly via positive modulation of the AP-1 DNA binding and transcriptional activity. Preliminary data obtained using an RNA anti-sense strategy, together with data obtained elsewhere, suggest that human Pwp1 might have a role in the regulation of cell growth.

To my father

This thesis is dedicated to my father, who unfortunately is no longer here to read it. I want to thank him, for it was he who advised me to study biology. Most likely without his help I would not be at this important stage of my career.

"Papa' " you were and always will be a role model of hard work, professionalism and great humanity. My decision to dedicate this thesis to you comes from my infinite and probably unsaid love for you.

With all my heart, grazie papa'!

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LIST OF CONTENTS

Title

Abstract

Dedication

Acknowledgments

List of contents

List of figures

Abbreviations

	Pages
<u>Chapter 1 - General introduction: multi-drug resistance mechanisms</u>	
Overview	2
1.1 Known multi-drug resistance mechanisms	3
Multi-drug resistance	3
P-glycoprotein (P-gp)	3
Multi-drug resistance protein 1 (Mrp1)	5
The glutathione pathway	5
The apoptotic pathway and p53	6
1.2 Novel determinants of multi-drug resistance in fission yeast	9
Fission yeast <i>pap1</i> ⁺	9
Fission yeast <i>pad1</i> ⁺	10
<i>pad1</i> ⁺ and drug resistance	12
Fission yeast <i>crm1</i> ⁺	13
<i>crm1</i> ⁺ and drug resistance	14
Human <i>CRM1</i>	14
Human <i>JAB1</i>	15
Fission yeast <i>int6</i> ⁺	16
General Discussion	17

Chapter 2 - Materials and methods

DNA manipulation	19
Polymerase chain reaction (PCR)	
Agarose gel electrophoresis	
DNA Sequencing	
DNA gel purification	
DNA ligation for cloning	
Cloning vectors for PWP1 and POH1	
Bacterial manipulation	24
Bacterial Electro-transformation	
Colony PCR	
Plasmid DNA preparations	
Preparation of GST-PWP1 fusion protein for raising polyclonal antibody	
Peptide conjugation for antibody preparation	
Fission yeast methods	29
Cell growth	
Measurement of cell concentration	
DNA transformation	
Expression of human Pwp1	
Protein extraction for immunoblot analysis	
Drug resistance assay	
Cell culture methods	31
Cell lines and media	
Transfection	
CD2 selection by magnetic beads	
Generation of stable transfectants	
Flow cytometry	
Annexin V binding assay	
Immunofluorescence	
Dual luciferase reporter assay	

Drugs sensitivity assays	37
Clonogenic survival assay	
MTS cytotoxicity assay	
Protein methods	38
SDS-PAGE	
Whole cell extracts for immunoblot analysis	
Immunoblot analysis	
Enhanced chemiluminescence (ECL) detection	
Membrane re-probing	
Immunoprecipitation	
Whole cell extracts	
Nuclear extracts	
End-labelling of double strand oligonucleotides	
Electrophoretic mobility shift assay	
<i>In vitro</i> degradation assay	
Purification of His ₆ -tagged c-Jun	
Sucrose density gradient centrifugation	
Yeast two-hybrid screen	46
Plasmids	
Lithium acetate transformation	
The interaction hunt	
Plasmid rescue	

Chapter 3 - Further characterization of the 26S proteasome subunit P_{oh1}

Overview	51
Introduction	
<i>POH1</i>	52
<i>POH1</i> in fission yeast	54
<i>POH1</i> in mammalian cells	54
The 26S proteasome complex	55
The 20S proteasome	56

The 19S regulatory particle	57
The base complex	57
The lid complex	598
Sub-cellular localization of the 26S proteasome	62
The lid, COP9 signalosome and eIF3 complexes	62
Results	
Constitutive stable expression of <i>HA-POH1</i> in COS-7 monkey cells	67
HA-Poh1 stable expression leads to drug resistance	69
Association of Poh1 with the 26S proteasome complex	73
Sub-cellular localization of HA-Poh1	79
Discussion	86
 <u>Chapter 4 - HA-Poh1 inducible expression in human cells</u>	
Overview	92
Introduction	
The tetracycline/doxycyclin mammalian inducible expression system	92
Results	
<i>HA-POH1</i> inducible expression systems used	97
Inducible expression of HA-Poh1 in human cells	101
Inducible expression of HA-Poh1 confers pleiotropic drug resistance in human cells	107
 <u>Chapter 5 - HA-Poh1 and AP-1 transcription factors</u>	
Overview	111
Introduction	
Activating protein 1 (AP-1)	111
c-Jun degradation pathways	116
AP-1 and drug resistance	118
Results	
HA-Poh1 increases the activity of AP-1 transcription factors	119
Ubiquitinylation of AP-1 in HA-Poh1 expressing cells	125
HA-Poh1 co-immunoprecipitates with c-Jun	131

HA-Poh1 overexpression increases c-Jun protein stability	134
Discussion	138

Chapter 6 - The human Pwp1 WD-repeat protein and drug sensitivity

Overview	145
Introduction	
The WD-repeat family	146
<i>S.cerevisiae PWP1</i>	148
Human <i>PWP1</i>	149
<i>S.pombe pwp1</i> ⁺	150
Results	
Human <i>HA-PWP1</i> in fission yeast	151
Stable expression of hPwp1 in mammalian cells	154
hPwp1 overexpression confers pleiotropic drug sensitivity	159
Discussion	164

Chapter 7 - Further characterization of the human Pwp1 protein

Overview	168
Results	
Sub-cellular localization of HA-hPwp1 in COS-7 cells	169
HA-hPwp1 overexpression increases AP-1 activity	171
Anti-sense RNA expression strategy	177
Human Pwp1 interacting partners	179
Discussion	185
 <i>General conclusions and further directions</i>	 191
 <i>Bibliography</i>	 195

Appendix

- Sequences:	215
- Primers	
- AP-1 oligonucleotides	
Peptides	
- Vectors:	217
- Vectors used for cloning	
Other vectors used	
- Antibodies:	218
Primary	
Secondary	
- Vector maps	219

LIST OF FIGURES

Figure		Page
2.1	The stress response in fission yeast	11
3.1	Poh1 protein sequence alignment with fission yeast Pad1, human Jab1 and S12/p40	53
3.2	The 26S proteasome complex: assembly of the core (20S) proteasome with the 19S regulatory complex	58
3.3	Schematic representation of the ubiquitin-dependent degradation pathway	61
3.4	Three multiprotein complexes containing PCI and MPN domains	63
3.5	Evolutionary origin of the 26S proteasome lid and the COP9 signalosome	66
3.6	Selection of CD2-expressing cells	68
3.7	HA-Poh1 stable overexpression in COS-7 polyclonal pool and in two clones	71
3.8	HA-Poh1 stable overexpression confers drug resistance	72
3.9	HA-Poh1 overexpression in clones 2 and 25 confers resistance to cisplatin and Doxorubicin	74
3.10	Overexpressed HA-Poh1 is incorporated into low molecular weight complexes	75
3.11	Exogenous HA-Poh1 co-precipitates with the proteasome core subunit C3	77
3.12	Most of the exogenous HA-Poh1 is not proteasome-associated	78
3.13	EGFP and EGFP-Poh1 protein detection in COS-7 cells	80
3.14	Transiently expressed EGFP-Poh1 is localised in the cytoplasm of COS-7 cells	80
3.15	EGFP localization in COS-7 cells	82
3.16	Exogenous HA-Poh1 is localized in the nucleus and the cytoplasm of COS-7 cells	84
3.17	Endogenous and exogenous Poh1 are present both in the soluble and in the nuclear enriched fractions	85

4.1	The tetracycline inducible system	95
4.2	The reverse tetracycline transactivator	96
4.3	The doxycyclin inducible system for HA-<i>POH1</i> gene expression	99
4.4	Regulation of HA-Poh1 inducible expression	100
4.5	Doxycyclin inducible expression of HA-Poh1 in stably transfected human U2-OS cells	102
4.6	Doxycyclin dose response curve of HA-Poh1 expression in U2-OS cells	104
4.7	Time course of HA-Poh1 expression in doxycyclin inducible stable clones	105
4.8	Endogenous and exogenous HA-Poh1 are present both in the soluble and in the nuclear enriched fractions of human cells	106
4.9	Treatment with doxycyclin does not affect the susceptibility of U2-OS cells to other drugs	108
4.10	Inducible expression of HA-Poh1 confers drug resistance in human cells	109

5.1	AP-1 transcription factors: stimuli and responses	113
5.2	Regulation of c-Jun activity	114
5.3	c-Jun regulation by JNK	115
5.4	HA-Poh1 overexpression leads to increased AP-1 binding activity and c-Jun protein level in COS-7 cells	121
5.5	HA-Poh1 overexpression leads to up-regulation of both AP-1 binding activity and c-Jun and c-Fos protein levels in COS-7 cells	123
5.6	Inducible expression of HA-Poh1 up-regulates AP-1 protein levels and binding activity in human cells	124
5.7	Constructs used for AP-1-dependent luciferase reporter assay	126
5.8	HA-Poh1 overexpression increases AP-1 transcriptional activity	126
5.9	HA-Poh1 overexpression does not affect general protein ubiquitinylation in COS-7 cells	129
5.10	Ubiquitinylation level of c-Jun in human cells overexpressing HA-Poh1	130

5.11	Ubiquitinylation levels of c-Jun and c-Fos in human cells overexpressing HA-Poh1	132
5.12	The ubiquitinylation level of c-Jun is not affected by overexpression of HA-Poh1	133
5.13	Exogenous HA-Poh1 co-immunoprecipitates with c-Jun in human cell extracts	135
5.14	HA-Poh1 overexpression increases c-Jun protein stability	137
5.15	Two models proposed for AP-1 regulation by HA-Poh1 in mammalian cells	143

6.1	Protein sequence alignment of PWP1 homologues from yeast to human cells	152
6.2	Expression of human HA-Pwp1 in <i>S.pombe</i> cells	153
6.3	Expression of human Pwp1 in <i>S.pombe</i> does not confer pleiotropic drug resistance	154
6.4	Stable expression of human HA-Pwp1 in mammalian cells	157
6.5	Expression of GST-Pwp1 recombinant protein in <i>E.coli</i>	158
6.6	Detection of Pwp1 protein in COS-7 cells using the rabbit polyclonal anti-Pwp1 antibody CN35	160
6.7	Stable expression of hPwp1 confers pleiotropic drug sensitivity in mammalian cells	162
6.8	The HA epitope tag fused to the <i>PWP1</i> gene is not responsible for the drug sensitivity phenotype	163

7.1	Stably overexpressed HA-Pwp1 in COS-7 cells is localized throughout the nucleus and the cytoplasm	169
7.2	Transiently expressed EGFP-Pwp1 is cytoplasmic	172
7.3	Treatment with leptomycin B partially changes the localization pattern of transiently expressed EGFP-Pwp1	173
7.4	Overexpression of HA-Pwp1 increases AP-1 DNA binding activity	175
7.5	AP-1 transcriptional activity is increased by overexpression of HA-Pwp1	176
7.6	The RNA anti-sense strategy	178

7.7	Anti-sense Pwp1 cells show a slow growth phenotype	180
7.8	Anti-sense Pwp1 cells have a high percentage of fragmented nuclei	180
7.9	The yeast two-hybrid screen: the interaction hunt	184

ABBREVIATIONS

A _{xyz}	Absorbance at a certain (xyz) optical density
aa	amino acid
ABC	ATP-binding cassette
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per million
dH ₂ O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
Dox	doxycyclin
dsDNA	double stranded DNA
ECL	enhanced chemiluminescence
E.coli	Escherichia coli
EDTA	ethylenediaminetetra-acetic acid
EGFP	enhanced green fluorescent protein
EMM	Edinburgh Minimal Medium 2
EMSA	electrophoretic mobility shift assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
<i>g</i>	standard gravitational constant
G418	geneticin sulphate
GST	glutathione-S-transferase

HA	haemagglutinin
hr	hour
HRP	horseradish peroxidase
HSV	Herpes Simplex Virus
ICRF	Imperial Cancer Research Fund
IPTG	isopropyl- β -D-thiogalactopyranoside
JNK	c-Jun NH ₂ -terminal kinase
kb	kilobase pair
kDa	kilodaltons
KLH	Keyhole Limpet Haemocyanin
LTR	long terminal repeat
LB	Luria-Bertani broth
MAPK	mitogen-activated protein kinase
MCP-21	multicatalytic protease antibody 21
min	minute
mRNA	messenger RNA
MW	molecular weight
NES	nuclear export signal
NLS	nuclear localization signal
<i>nmt</i>	<i>no</i> message in thiamine
NP-40	nonidet-40
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
P-gp	P-glycoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	Phorbol-12-myristate 13-acetate
RLA	relative luciferase activity
RNA	ribonucleic acid
RNAse	ribonuclease

rpm	revolutions per minute
SD	synthetic dextrose minimal medium
SDS	sodium dodecyl sulphate
ssDNA	single strand DNA
TBE	tris/borate/EDTA
TBS	tris buffered saline
Tc	tetracyclin
TE	tris/EDTA/ buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TRE	tetracyclin responsive element
trp	tryptophane
Tris	tris[hydroxymethyl]aminomethane
tTA	tetracyclin transactivator
UCN-01	7-hydroxystaurosporine
WT	<i>wild-type</i>
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
YE5S	yeast extract with five supplements (leucine, histidine, adenine, uracil, and lysine hydrochloride)
YPD	yeast extract peptone dextrose

Chapter 1

General introduction: multi-drug resistance mechanisms

Overview

This thesis describes the characterization of the 26S regulatory proteasome subunit Pih1 in relation to the multi-drug resistance phenotype in mammalian cells. This study was carried out using constitutive and inducible expression systems. Additional work concerns the characterization of the human *PWWP1* gene, which is the homologue of the fission yeast multi-drug resistance gene *pwp1*⁺, and the phenotype of multi-drug sensitivity surprisingly conferred by the overexpression of the Pwp1 protein in mammalian cells.

The first part of the chapter describes the impact of multi-drug resistance on cancer therapy and the mechanisms that are thought to be involved in this phenotype. The second part of the chapter focuses on some new drug resistance determinants that have recently been identified in fission yeast.

1.1 Known multi-drug resistance mechanisms

Multi-drug resistance

Radiotherapy and chemotherapy are the main non-surgical approaches to the treatment of cancer. The main aim of these anti-cancer agents is to cause cancer cell death. Ionizing radiation and many chemotherapeutic agents induce DNA damage or cause disruption in the DNA metabolism. Different types of DNA damage may lead to the activation of a common signal that initiates the apoptotic cell death. The capacity of the cancer cells to detect cellular damage and to activate their own cell death programme is the principle desired result of a successful response to cancer therapy (Lowe et al. 1993).

Unfortunately, many solid tumours fail to respond to the therapies as resistant cell populations arise from the originally responsive malignancy. In some tumour type, such as breast cancer, cells appear to be intrinsically resistance to cytotoxic drugs, even before they have been exposed to the therapy.

Cancer cells are frequently able to develop cross-resistance to several unrelated drugs when treated with a specific genotoxic agent.

Several mechanisms have been identified that can contribute to this resistance phenotype.

The P-glycoprotein (P-gp) pump

P-glycoprotein (P-gp) is a plasma-membrane energy-dependent pump belonging to the ATP-binding cassette (ABC) super-family of transporters. It is a 170 kDa protein (p170) encoded by the multi-drug resistance gene 1 (*MDR-1*) (Chen et al. 1986) (Gros et al. 1986). P-gp is composed of two halves, each containing six

transmembrane domains and an ATP binding domain. ATP binding and its hydrolysis are both essential for the proper functioning of the protein as a pump.

An elevated level of P-gp (p170) in mammalian cells, due to elevated gene expression, with or without gene amplification of the *MDR-1* gene, is a cause of resistance to a wide range of chemotherapeutic drugs (Ambudkar et al. 1999). The expression of functional P-gp leads to an energy-dependent efflux of cytotoxic drugs out of the cytosol, causing a reduction of the steady level of the drug inside the cell and a drug resistance phenotype. The currently accepted model for P-gp function is the “flippase” model. In the model the pump intercepts the drug as it moves through the lipid membrane and flips the drug from the inner to the outer leaflet and into the intracellular medium (reviewed by Johnstone et al. 2000). Unfortunately, it is still not very clear how the P-gp is able to recognise such a wide range of different drugs, such as anticancer drugs, HIV protease inhibitors, cyclin and linear peptides and other cytotoxic agents (Ambudkar et al. 1999).

P-gp is also associated with a volume-regulated chloride channel activity and this role seems to be distinct from its ability to act as drug transporter. In fact, the tissue distribution of P-gp expression suggests a primary function as a chloride channel, instead of, or in addition to, its activity as multi-drug transporter (Gottesman and Pastan 1993).

A bacterial ABC transporter, LmrA, has been described to have physiological and functional properties that are almost identical to those of the P-gp (van Veen et al. 1998). In fact, the two energy-dependent transporters are functionally interchangeable, underlying the high degree of conservation from bacterial to human cells.

The multi-drug resistance protein 1 (Mrp-1)

The multi-drug resistance protein (Mrp1), another transporter belonging to the ABC protein family, was cloned in 1992 (Cole et al. 1992) from a multi-drug resistant human cell line. Up-regulation of MRP1 RNA expression or the post-translational modification of the protein may lead to multi-drug resistance to many cytotoxic drugs, such as doxorubicine, daunorubicin, vincristine, vinblastine and etoposide. No resistance to cisplatin was shown. It has been suggested that Mrp-1 could function as a pump, not only increasing the efflux of the drug through the plasma membrane but also by pumping the drugs into the cytoplasmic vesicles with the subsequent removal of the cytoplasmic vesicles from the cell by exocytosis (Broxterman et al. 1995).

Furthermore, it has been also suggested that the transport of positively charged drugs, such as vincristine, daunorubicine and rhodamine 123, through the Mrp-1 pump, is regulated by co-transport with glutathione (Broxterman et al. 1995).

The glutathione pathway

Glutathione is the main non-protein thiol in eukaryotic cells. The main role of glutathione and glutathione-dependent enzymes is to protect the cells against toxicity caused by chemical compounds or by normal metabolic pathways. Thus, it is considered the primary defence of the cell against oxidative stress. The glutathione pathway is involved in several cellular processes, such as drug detoxification, drug transport, modulation of DNA signalling and apoptosis (reviewed by McLellan and C.R. 1999).

As glutathione and glutathione-dependent enzymes are essential for cell survival following toxic insult, it is clear that they are also linked to a resistance phenotype of

human tumours to the drug therapy (Hayes and Wolf 1990). There are several mechanisms by which glutathione and glutathione-dependent enzymes might lead to drug resistance (McLellan and C.R. 1999).

- Detoxification by the conjugation of the anti-cancer drugs with glutathione and their subsequent export by an energy-dependent mechanism
- Free radical scavenging
- Reduction of peroxidases and detoxification of the products of the lipid peroxidation
- Export of un-conjugated drugs by glutathione-dependent transport through the multi-drug resistance protein 1 and 2 (Mrp-1 and Mrp-2)
- Regulation of signalling pathway by redox status. Redox sensitive proteins include AP-1, NFκB, MAP kinases, SAP kinases, c-Jun and c-Fos
- Inhibition of apoptosis, the serine protease AP24 is inhibited by glutathione; a high level of the anti-apoptotic protein Bcl-2 causes an increase of the intracellular level of glutathione and promotes its nuclear localization

The apoptotic pathway and p53

Apoptosis, or programmed cell death, is a major mechanism for the maintenance of homeostasis of the body's systems under physiological conditions. After the apoptotic process has been triggered the cell undergoes a cascade of biochemical changes, which lead to its self-destruction.

The cytotoxicity of many anti-cancer drugs, which induce DNA damage or disrupt DNA metabolism, is attributable to activation of the genetic programme of cell death. The common capacity of various chemotherapeutic drugs to induce apoptosis

suggests that multiple mechanisms are able to activate the same pathway (Sen and D'Incalci 1992).

The tumour suppressor p53 is a transcription factor, also known as the "guardian of the genome" (Lane 1992), which by regulating apoptosis and checkpoints responses to DNA damage and other forms of genotoxic stress has a central role in the control of the genomic integrity. p53 is mutated or inactivated by some other mechanisms in most human tumours. p53-independent mechanisms of DNA damage-induced apoptosis and cell cycle arrest also exist (reviewed by Agarwal et al. 1998).

Genotoxic stress causes an increase of p53 protein level, its nuclear localization and sequence-specific DNA binding. As a result, p53 target genes, including Mdm2, Gadd53 and cyclin-dependent kinase inhibitor Cip1, are induced with consequent arrest at the G1, either transiently or prolonged, and G2 phase of the cell cycle in order to allow the DNA repair machinery to repair the damage before DNA replication or mitosis occur, or alternatively leading to the activation of apoptosis (Di Leonardo et al. 1994). The choice between cell cycle arrest and induction of apoptosis depends on multiple variables, including the cell type, the extent of DNA damage and the level of p53 itself in the cells. For example low levels of p53 promote cells survival in fibroblasts, underlying an anti-apoptotic function of p53 (Lassus et al. 1996). Furthermore, links between p53 and other key regulators of the cell cycle and apoptosis, such as pRb, c-Myc, Bax and Bcl-2, have also been demonstrated (Agarwal et al. 1998; Miyashita et al. 1994).

Given that p53 has been shown to have a key role in determining the choice between cell survival and apoptosis, tumour-associated p53 mutations might be expected to contribute to increased cross-resistance of the cell population to DNA damaging agents. Thus one of the mechanisms by which cancer cells acquire drug and/or

radiation resistance is through the modulation of the apoptotic pathway (Lowe et al. 1993).

1.2 Novel determinants of multi- drug resistance in fission yeast

The fission yeast *Schizosaccharomyces pombe* is an established genetic model that has been used to investigate many complex eukaryotic processes such as mitosis and checkpoint regulation (Nurse 1990). In order to identify new determinants of multi-drug resistance that might be conserved in human cells, over the last few years genetic studies in fission yeast have also addressed this area of biology.

Fission yeast *pap1*⁺

Fission yeast *pap1*⁺ is a non-essential gene encoding an AP-1-like transcription factor containing a leucine zipper motif (Toda et al. 1991) (reviewed by Toone and Jones 1999). The basic domain that is located in the proximity of the leucine repeats is similar to the basic domain found in c-Jun, c-Fos and GCN4 transcription factors. The overall sequence homology in this region among these proteins is about 40%. Furthermore, the basic region is similar to the *S.cerevisiae* YAP1 transcription factor sequence (yeast AP-1 like gene I).

The purified recombinant Pap1 is able to bind specifically to the human collagenase (Col-AP1) or the SV-40 (SV-AP1) promoters, which both contain the AP-1 consensus binding sequence (Toda et al. 1991). When two of the consensus nucleotides are mutated, Pap1 is no longer able to form a DNA-protein complex.

The overexpression of *pap1*⁺ from a multicopy plasmid leads to resistance to staurosporine in fission yeast while on the contrary the *pap1*⁺-deleted cells are hypersensitive to staurosporine (Toda et al. 1991). Pap1 is regulated by the fission yeast stress-activated MAP kinase Sty1 (Toone et al. 1998). After exposure of fission yeast cells to cytotoxic agents and/or to oxidative stress, Pap1 re-localises in the

nucleus with subsequent transcriptional activation of the target genes involved in the stress response. These include *hba1⁺/bfr1⁺* and *pmd1⁺* genes, encoding two energy-dependent ABC family transport proteins as well as other genes involved in multi-drug resistance (Toone et al. 1998). This process is Sty1-dependent and it is regulated at the nuclear export level, rather than the import level (Figure 2.1). The process is dependent on the Crm1 nuclear export factor and the Ran nucleotide exchange factor, encoded by the *crm1⁺* and the *dcd1⁺/pim1⁺* genes, respectively.

Fission yeast *Pad1⁺*

The fission yeast *pad1⁺* gene, for *pap1*-dependent transcriptional activator *L*, was first described in 1995 as a positive regulator of Pap1 (Shimanuki et al. 1995). The *pad1⁺* gene is essential for cell viability. *pad1⁺*-disrupted spores can germinate, but show altered chromosome structure where the nucleus of the null mutants is often abnormally located in the cell body. The chromosomal DNA is also abnormally deformed and the chromatin appears more compact in comparison with the wild-type cells. The arrested cells can also be unusually elongated. This evidence reveals critical roles of the *pad1⁺* gene both for chromosome integrity and for cell cycle progression (Shimanuki et al. 1995).

It was suggested that Pad1 might be involved in establishing the chromatin architecture during cell cycle progression and that it may regulate the maintenance of the chromosome structure through an indirect mechanism. In fact the cell cycle arrest in the null mutant cells could be a consequence of the formation of an abnormal chromosome structure (Shimanuki et al. 1995).

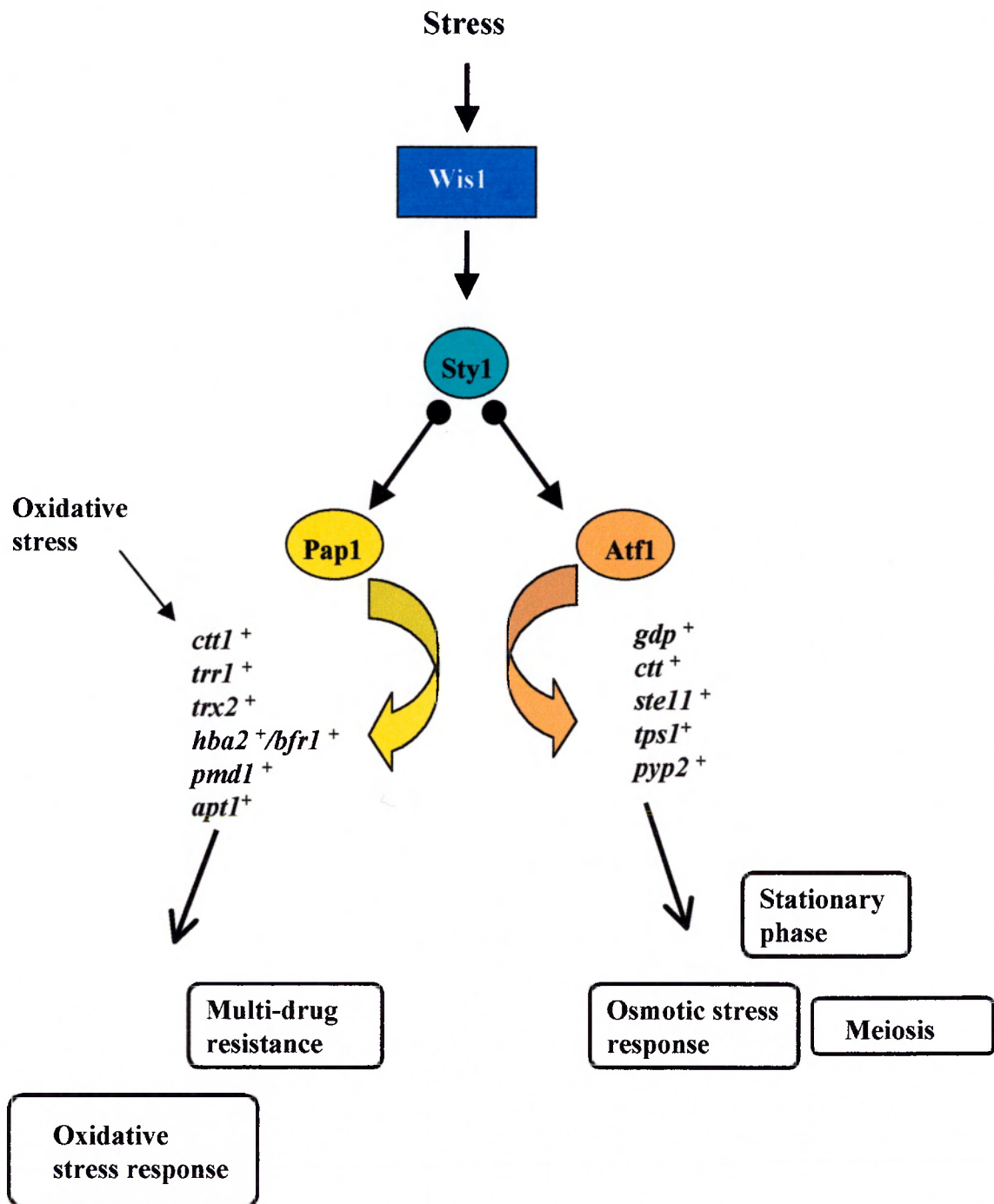


Figure 2.1 – The stress response pathway in fission yeast

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Subsequently it was shown that *pad1*⁺ encodes a regulatory subunit of the 26S proteasome in fission yeast (Penney et al. 1998). Pad1 co-purifies with the fraction containing the highest levels of the 26S proteasome activity. In support of these data, Pad1 co-immunoprecipitates with the 26S regulatory subunit Mts4 from crude yeast extracts (Penney et al. 1998).

The fission yeast 26S proteasome is essential for cell growth and loss of its function leads to the arrest of the cell cycle at metaphase (reviewed by Penney et al. 1998). Consistent with this phenotype, the temperature sensitive mutant *pad1-1* shows a growth arrest phenotype. Furthermore the *pad1-1* mutant, as well as the *mts2-1* mutant (which is defective in another proteasome regulatory subunit), shows an accumulation of high molecular weight ubiquitinated proteins, consistent with one of the main functions of the 19S regulatory complex being the recognition of the ubiquitinated substrates. Taken together, these results support the idea that Pad1 is a novel component of the 19S regulatory complex of the 26S proteasome in fission yeast.

***pad1*⁺ and drug resistance**

The initial isolation of *pad1*⁺ was as a gene lacking the 3' portion at the open reading frame encoding the last 29 amino acids, which conferred staurosporine resistance when overexpressed from a multicopy plasmid in fission yeast (Shimanuki et al. 1995). In the chromosome the *pad1*⁺ gene resides in close proximity to the *spk1*⁺ gene (staurosporine-resistance protein kinase I), which was initially thought to be the gene responsible for the staurosporine resistance (Toda et al. 1991). The resistance phenotype is dependent on the presence of *pap1*⁺ as *pap1*⁺-disrupted cells do not show any resistance. *pad1*⁺ overexpression appeared to cause activation of Pap1-

dependent transcription without affecting either mRNA or protein level of Pap1. As observed in *pap1*⁺-overexpressing cells, Pad1 induces the transcriptional activation of the target gene *apt1*⁺, which encodes the 25kDa molecular weight mass flavoprotein p25, while on the other hand the DNA binding ability of Pap1 was apparently not affected by Pad1 overexpression.

Given that Pad1 is a regulatory subunit of the 26S proteasome complex (Penney et al. 1998), it has been suggested that the truncated *pad1*⁺ gene could generate an abnormal protein that in some way disrupts the function of the 26S proteasome, resulting in the accumulation of proteins possibly involved in the drug resistance mechanism (Penney et al. 1998).

Fission yeast *crm1*⁺

The fission yeast *crm1*⁺ gene, for chromosome region maintenance 1, is an essential gene encoding a highly conserved 115 kDa protein related to the β -importin-like nuclear transport factors (Adachi and Yanagida 1989). *crm1*⁺ was originally identified through a cold-sensitive (*cs*) mutant during a microscopic screen of fission yeast cells stained with 4,6-diamidino-2-phenylindole (DAPI) in order to identify gene products essential for maintaining higher-order chromosome structure (Adachi and Yanagida 1989). At permissive temperature the *cs crm1* mutant cells showed pleiotropic phenotypes, including calcium hypersensitivity, staurosporine resistance and accumulation of p25 (Adachi and Yanagida 1989). *crm1*⁺ was subsequently described as a negative regulator of Pap1 (Toda et al. 1992).

Fission yeast *crm1*⁺ encodes an export factor involved in Ran-dependent nuclear export, as has already been shown for the homologous protein in *S.cerevisiae*,

Xenopus, *Drosophila* and human cells. The protein localises in the nucleus, but is also enriched in the nuclear periphery (Adachi and Yanagida 1989).

***crm1*⁺ and drug resistance**

Mutations of the *crm1*⁺ gene were shown to cause resistance to staurosporine and caffeine through increased Pap1-dependent transcription (Toda et al. 1992) with associated up-regulation of the expression of p25 expression. However, no increase in Pap1 expression level was observed. This suggested that the capacity of *crm1*⁺ to negatively regulate Pap1 activity is not dependent on any modulation of Pap1 protein level. It has since transpired that Crm1 controls the nuclear/cytoplasmic transport of Pap1 (Toone et al. 1998). Consistent with this role, *crm1* mutant cells show a nuclear accumulation of Pap1 with the consequent increased transcription of target genes involved in drug resistance.

Human CRM1

Human CRM1 was first described in 1997 as a 112 kDa soluble factor that interacts with the nuclear pore complex (NPC). The protein contains a domain of significant homology with importin- β , a cytoplasmic factor that interacts with the nucleoporin repeat regions (reviewed by Fornerod et al. 1997a; Fornerod et al. 1997b; Ossareh-Nazari et al. 1997). The NPC is a 125 MDa complex embedded in the nuclear envelope that mediates nucleocytoplasmic traffic in eukaryotic cells. Crm1 protein is localised in the nuclear pore as well as in the nucleoplasm. It is a dynamic protein, which can interact with multiple components of the NPC and move between the nuclear pore and the cytoplasm (Fornerod et al. 1997b).

Human *JAB1*

Human *JAB1* (c-Jun-activation domain binding 1) was identified through a two hybrid screen for proteins able to interact with the amino-terminal activation domain of c-Jun (Claret et al. 1996). Jab1 is able to interact with c-Jun, which is the major component of the heterodimeric AP-1 transcription factors, as well as with JunB but not with JunD or with v-Jun. Jab1 has also extensively sequence similarity to the product of the open reading frame (ORF) F37A4.5 from *C.elegans*, with *S.pombe* Pad1 and with the human Pad1 homologue, Poh1.

Jab1 is mainly localised in the nucleus and is able to increase the transcriptional activity of the AP-1 factors in mammalian cells. Furthermore, when Jab1 is overexpressed in fission yeast cells it confers drug resistance in a Pap1-dependent mechanism. In fact, Jab1 was the first mammalian co-activator shown to be able to modulate the expression of endogenous genes in fission yeast (Claret et al. 1996) and with Pad1 defined as a new family of eukaryotic co-activators able to positively modulate some members of the AP-1 family (Claret et al. 1996).

Recently Jab1 was identified as subunit 5 (CSN5) of the COP9 signalosome complex, also known as JAB1-containing signalosome, a multiprotein complex first described in plants but highly conserved in fission yeast and humans (see Introduction Chapter 3) (Seeger et al. 1998).

Recent data indicate that Jab1 is involved in a regulation of various cellular processes, including transcription, protein degradation, complex stabilisation and signalling. For example, Jab1 is able to bind the cyclin-dependent kinase inhibitor p27^{Kip1} with the subsequent translocation of the protein to the cytoplasm and an increase of p27^{Kip1} degradation by the 26S proteasome pathway (Tomoda et al. 1999). Jab1 also interacts with the cytoplasmic domain of the β_2 -subunit of the integrin

LFA-1 (Bianchi et al. 2000). Recently it has been shown that Jab1 binds the progesterone receptor (PR) and the steroid receptor coactivator 1 (SRC-1), with the subsequent stabilization of the complex (Chauchereau et al. 2000). These data suggest that in general Jab1 might regulate transcription by a mechanism that involves the bridging of receptors and coactivators.

Fission yeast *int6*⁺

Int-6 (p48) is a component of the human translation initiation factor eIF3. The murine *int-6* gene is a site of mouse mammary tumour virus (MMTV) insertion, which leads to the formation of a truncated Int-6 protein with tumorigenic potential (Marchetti et al. 1995). A fission yeast *int6*⁺ homologue has been recently identified through a screen aimed at identifying genes able to confer multi-drug resistance when overexpressed in *S.pombe* cells (Crane et al. 2000). Similarly to the phenotype described for *pap1*⁺ (Shimanuki et al. 1995), the *int6*⁺ drug resistance phenotype is dependent on the *pap1*⁺ gene and *int6*⁺ overexpression leads to an increase of Pap1-dependent transcription, without an increase in the Pap1 protein level or changes in its localisation. *int6*⁺ encodes a 57 kDa protein Int6, which appears to be part of the eIF3 complex as it co-immunoprecipitates with other components of the eIF3 complex. Furthermore, following sucrose gradient centrifugation Int6 was present in size fractions containing the 43S translation preinitiation complex.

int6⁺ is not an essential gene, but cells lacking the gene showed a slow growth phenotype. Normal growth was restored by the overexpression of the fission yeast *int6*⁺ gene, as well as the human homologue *INT6* (Crane et al. 2000).

General conclusions

It is apparent that a complex network of different pathways regulates the response of the cells to the cytotoxic effect exerted by chemotherapy and radiotherapy in cancer. In the last decades much effort from both experimental and clinical works have been focused on a better understanding of the mechanisms through which previously described mechanisms as well as recently identified drug resistance determinants once inactivated or deregulated can interfere with the cell death response induced by toxic insult and can subsequently lead to resistance to the therapy.

General aim of the work described in this thesis was to characterize two human genes, whose counterparts in fission yeast confer multi-drug resistance, and their mechanism of action in mammalian cells, in order to possibly elucidate novel pathways or downstream effectors underlying the pleiotropic drug resistance often observed in cancer.

Chapter 2

Materials and Methods

1.0 DNA MANIPULATION

Polymerase chain reaction (PCR)

Template DNA was diluted to a concentration of 100 ng/μl in dH₂O. The PCR mixture was prepared as follows:

Deoxynucleotide mix [5 mM each]	2 μl
Expand PCR buffer 10x	5 μl
Oligonucleotide primer [40 μM]	2.5 μl
Template DNA	1 μl
Expand polymerase (Roche)	2.5 units
dH ₂ O	to 50 μl

Thermal cycling was performed in a GeneAmp PCR system 9700 machine (Perkin-Elmer) as follows: 94°C for 5 minutes, 20-30 cycles of 94°C 30 seconds, annealing temperature 55-65°C for 30-60 seconds, 72°C for 30 seconds - 2 minutes (depending on the length of the sequence to amplify) and 72°C for 10 minutes before chilling at 4°C.

Agarose gel electrophoresis

DNA samples were mixed with 0.2 volume of 6x loading buffer (30% (w/v) sucrose, 0.1 mM EDTA, 0.01% (w/v) bromophenol blue) and loaded onto 1-1.2 % agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 20 mM EDTA pH 8.0) containing 0.5 μg/ml of ethidium bromide. Electrophoresis was carried out at a constant voltage of 2-10 V/cm and the DNA was visualised using a UV light box.

DNA Sequencing

DNA sequencing was performed using the dideoxynucleotide chain termination method. The sequencing reaction was performed in 10 µl final volume containing 3.2 picomoles of oligonucleotide primer and 10-50 nanograms of template DNA using ABI PRISM dRhodamine dye terminator reagent (Perkin-Elmer). After thermal cycling (96°C for 10 seconds, 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 50°C for 4 minutes), the DNA was ethanol precipitated and resuspended in bromophenol blue-containing buffer and heated at 98°C for 5 minutes. The samples were run on a semi-automated sequencer (ABI PRISM 377) with the assistance of Reg Boone and James Beesley and analysed using the Sequencher 3.0 software (Manufacturer).

DNA purification from agarose gels

DNA was purified from agarose gels using a gene-clean II kit (BIO 101) according to the manufacturer's instructions. After electrophoresis, the DNA band of interest was excised from the gel under long-wave UV light and weighted. 3 volumes of 6 M NaI solution was added to the tube, which was then incubated for 5 minutes at 55°C. 5-10 µl of glass milk were added and incubated for 5 minutes on ice. After three washes with 50% ethanol solution, the DNA was eluted in 20 µl of TE.

DNA ligation for cloning

After gel purification, linearised vectors and inserts were ligated at molar ratios of 1:3 and 1:5 using 1U of T4 ligase in 1x T4 ligation buffer in a total reaction volume of 10 µl. Ligation reactions were performed at 16°C overnight or at room temperature for 1-2 hours.

Cloning vectors

▪ Human *PWPI*

- Human *PWPI* cDNA was cloned into the fission yeast pREP3X vector, which drives the expression of the gene from the thiamine-repressible *nmt1* promoter (Maundrell 1993). A triple haemagglutinine (HA) epitope tag was previously introduced between *XhoI* and *Sall* sites in the polylinker (by Randa Craig). Human *PWPI* was amplified from a human cDNA kindly provided by Dr. Celis using the sense *PWPI* 5' primer containing a *Sall* restriction enzyme site and the reverse *PWPI* 3' primer containing a *NotI* site. *PWPI* open reading frame was fused at the COOH-terminus of the HA epitope tag, and the vector was named pREP3X/HA-*PWPI*. The cDNA was sequenced using 4 different primers: *PWPI* 5' mutchk, *PWPI* 3' Pst, *PWPI* 5'INT and the two primers used for cloning, (*PWPI* 5' and 3') in order to cover the length of the entire gene (1596 bp).

- *HA-PWPI* was cloned in pRETRO-Off vector (Clontech), a Moloney murine leukaemia virus (MoMuLV)-derived retroviral vector expressing the tetracycline-controlled transactivator (tTA) from the SV-40 promoter. The vector was modified in order to obtain a better multicloning site (MCS) as follows: the vector was digested with *XhoI* (+1) and treated with the Klenow enzyme in order to blunt the DNA ends and destroy the restriction enzyme site on re-ligation. Subsequently, the vector lacking *XhoI* was digested with *NotI* and *BamHI* and ligated to a double stranded linker, obtained by annealing of the oligonucleotides linker 5' and linker 3', with *NotI* and *BamHI* half-sites at its ends. The result was a MCS containing *XhoI*, *MluI*, *HpaI*, *ClaI* *NotI* and *BamHI*, where the *NotI* site from the original vector was destroyed. *HA-PWPI* was digested with *XhoI* and *NotI* from pREP3X/HA-*PWPI* and ligated into the multi-cloning site (MCS) of the vector under the control of the tetracycline

responsive element (TRE). The correct insertion of the gene in the vector was checked by sequencing, using the sense pRETOFF 5' and the reverse pRETOFF 3' primers, which are complementary to the multi-cloning site sequence of the vector.

- In order to clone *HA-PWP1* into the pcDNA3.1+ mammalian expression vector (Invitrogen), the gene was amplified by PCR using the sense PWP1 *Hind/Sal* primers, which contains *HindIII* and *Sall* restriction enzyme sites, and the reverse PWP1 3' primer with a *NotI* site. The vector and the PCR product were digested *HindIII* and *NotI* and ligated.

- In order to clone *PWP1* without an HA epitope tag at the 5' of the gene, the sense primer PWP1 ATG (no tag), containing a *HindIII* site, and the reverse primer PWP1 3', containing a *NotI* site, were used. The PCR product was digested with *HindIII* and *NotI* and cloned into pcDNA3.1+, which was digested with the same restriction enzymes. The PWP1 sequence was confirmed as described above.

- *PWP1* was cloned in the anti-sense orientation into pcDNA3.1- (Invitrogen), which contains the same MCS as pcDNA3.1+, but in the opposite orientation, using *HindIII* and *NotI* sites.

- In order to raise polyclonal antibodies against Pwp1, the cDNA was cloned into pGEX-4T-3 (Pharmacia Biotech) at the 3'-end of the GST sequence and expressed as a GST-PWP1 fusion protein in *E.Coli* BL21. pREP3X/HA-PWP1 was digested with *Sall* and *NotI* at the 5' and the 3' ends of the cDNA, which was cloned into pGEX-4T-3 digested with the same enzymes.

- For localisation studies *in vivo*, *PWP1* was also cloned at the COOH-terminus of the EGFP (enhanced green fluorescent protein) coding sequence in the pEGFP-C1 vector (Clontech). The fusion protein is expressed under the control of the immediate early promoter of the human cytomegalovirus (CMV). The pREP3X/HA-

PWP1 and the pEGFP vector were both digested with *Sall* and *BamHI* and vector and insert were ligated.

- *HA-PWP1* was also cloned into the IPTG inducible vector, pOPRSVI (Stratagene), previously modified (by C. Norbury) with *XhoI*-*ClaI*-*NotI* cloning sites, using *XhoI* and *NotI* sites at the 5' and the 3' ends of the cDNA, respectively.

- In order to identify possible interacting targets of the Pwp1 protein, a two hybrid screen was performed. The *PWP1* cDNA was cloned into the pEG202 vector. In order to obtain *PWP1* sequence in frame with *LexA*, the gene was first digested from pREP3X-PWP1 plasmid and cloned into the pIRES2-EGFP vector (Clontech) using *Sall* and *BamHI* sites. PWP1 was then digested from pIRES2-EGFP/PWP1 with *EcoRI* and *BamHI* and ligated into pEG202 vector digested with the same enzymes.

▪ Human *POH1*

- *POH1* was previously cloned into pcDNA3 (Invitrogen) using the *HindIII* and *NotI* sites at the 5' and the 3' ends of the cDNA, respectively, with a single HA epitope tag at the 5' end of the cDNA (Spataro et al. 1997).

- In order to express the protein through an inducible system, *POH1* was cloned into pOPRSV, previously modified (by C. Norbury) with *XhoI*-*ClaI*-*NotI* cloning sites. *POH1* was excised from pREP3X/HA-*POH1* and cloned into pOPRSVI using the *XhoI* and *NotI* sites.

- *HA-POH1* was cloned into pUHD10-MCS for inducible expression in human cells. A PCR fragment was generated using the synthetic sense *POH1NotI* and the reverse *POH1EcoRI* primers. In addition, a single HA sequence was fused in frame with the first codon at the 5' end of the cDNA. The PCR fragment and pUHD10-MCS vector

were digested with *NotI* and *EcoRI* enzymes and ligated together. The gene was then sequenced.

- *POH1* was also cloned at the COOH-terminus of the EGFP sequence into pEGFP-C1 (Clontech). *POH1* was amplified by PCR using the *POH1Sal5'* and the *POH1Xma3'* primers. Both the PCR product and the vector were digested with *Sall* and *XmaI* and ligated.

2.0 BACTERIAL METHODS

Bacterial electro-transformation

A) Preparation of electro-competent cells

A single colony of *E.coli* DH10 β (*F⁻ araD139 (ara, leu)7697 Δ lacX74 galU galK mcrA Δ (mrr – hsdRMS – mcrBC) λ -rpsLdeok Φ 80dlacZDM15 endA1 nupG recA1*) was inoculated into 5-10 ml of LB medium and grown overnight at 37°C. The culture was diluted into 400 ml of medium and the bacteria were grown at 37°C with vigorous shaking to an $A_{600} = 0.5-0.8$. The flask was then chilled on ice for 15-30 minutes and the bacterial culture was centrifuged at 5000 rpm in a JA10 rotor for 15 minutes at 4°C. The pellet was washed twice with 400 ml and 200 ml, respectively, of ice-cold sterile distilled water and centrifuged for 15 minutes at 5000 rpm in a JA10 rotor at 4°C. The pellet was resuspended in 10 ml of 20% glycerol and spun down in a JA20 rotor. Finally the cells were resuspended in 1-2 ml of 10% glycerol. The cells were frozen in aliquots (50-100 μ l) on dry ice and then stored at -70°C up to 6 months.

B) Electro-transformation

The Gene Pulser electroporator (BIO-RAD) was set to 25 μ F capacity, 2.5 kV and the Pulse Controller unit to 400 Ω . An aliquot of competent cells was gently thawed

and kept on ice. 0.8-1µl of DNA was added to 50 µl competent cells, transferred to a 0.2 cm electroporation cuvette and the cells/DNA suspension was gently shaken to the bottom of the cuvette. A single pulse was applied to the cells with a time constant of 10 msec and 200 µl of LB medium was added immediately to the cells, which were plated on LB agar (+ selective antibiotic) plates. The plates were incubated overnight at 37°C.

Colony PCR

Each colony was inoculated into 0.5 ml of LB medium, containing the appropriate antibiotic and grown for about 1-2 hours. 5 µl of the culture was used for PCR reaction in a 50 µl final reaction volume. In the first 5 minutes at 94°C the bacteria cells were lysed and the DNA became available for the PCR reaction (20-25 cycles). 10 µl from each PCR reaction was analysed on 1-1.5% agarose gels and positive colonies analysed by mini-prep.

Plasmid DNA preparations

a) Small scale preparation of plasmid DNA (QIAGEN mini-prep)

After DNA transformation, a single bacterial colony was inoculated into 5 ml of LB medium, containing the appropriate antibiotic (Ampicillin [100 µg/ml] or Kanamycin [50 µg/ml]) and grown overnight at 37°C. 2-3 ml of the culture was spun down at maximum speed in an eppendorf centrifuge for few minutes and the bacterial pellet was resuspended in 300 µl of buffer P1 (Qiagen) (50mM Tris-HCl pH= 8.0, 10mM EDTA and 100µg/ml RNase A). 300 µl of buffer P2 (200 mM NaOH, 1% SDS) was added, mixed gently by inversion and incubated for 5 minutes at room temperature. 300 µl of chilled buffer P3 (3.0 mM potassium acetate pH= 5.5) was added and

incubated for 10 minutes on ice. The lysate was spun down for 10 minutes at 4°C at 13.000 rpm in a eppendorf centrifuge and the supernatant was precipitated by centrifugation at 13.000 rpm in an eppendorf centrifuge with 0.8 volume of propanol-2-ol at 4°C for 20-30 minutes. The precipitated DNA was washed with 70% ethanol and allowed to dry. The DNA was resuspended in 20 µl of TE (pH 8.0). For a cleaner DNA preparation, after incubation with the solutions P1, P2 and P3 and centrifugation, the supernatant was passed through a Qiagen column tip-20, which was previously equilibrated with the buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% propanol-2-ol and 0.15% Triton X-100). The column was then washed 4 times with 1 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0 and 15% propanol-2-ol); DNA was eluted with 0.8 ml of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5 and 15% propanol-2-ol), collected in an eppendorf tube and precipitated using 0.7 volumes (0.57 ml) of propanol-2-ol at room temperature centrifugation at maximum speed in an eppendorf centrifuge at 4°C for 30 minutes. The pellet was then washed with 70% ethanol and allowed to dry. The DNA was resuspended in 20 µl of TE or dH₂O.

b) Large scale preparation of plasmid DNA (QIAGEN maxi-prep)

A single bacterial colony was inoculated into 5 ml of LB containing the appropriate antibiotic and allowed to grow for 6-8 hours at 37°C. The culture was diluted into 400 ml of the same medium and grown overnight with vigorous shaking at 37°C. The bacteria were spun down at 5000 rpm in a J10 rotor for 15 minutes and the bacterial pellet was resuspended in 10 ml of buffer P1 (as before). 10 ml of buffer P2 were added and the solution was mixed gently by 5-8 times inversion and incubated for 5 minutes at room temperature. 10 ml of buffer chilled P3 were added, mixed gently and incubated on ice for 15 minutes. The lysates was spun down at 12.000 rpm using

a J14 rotor at 4°C for 30 minutes and the supernatant was applied to a QIAGEN-tip 500, which was previously equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% propanol-2-ol and 0.15% Triton X-100). Then, the column was washed twice with 30 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0 and 15% propanol-2-ol). The DNA was eluted with 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5 and 15% propanol-2-ol) and the eluate was collected in a 30 ml tube. The DNA was precipitated by adding 0.7 volumes of room temperature propanol-2-ol and centrifuged at 12.000 rpm in a J20 rotor for 30 minutes at 4°C. The pellet was washed once or twice with 70% ethanol, allowed to dry and redissolved in TE pH 8.0.

Preparation of GST-PWP1 fusion protein for raising polyclonal antibodies

E.coli strain BL21 was transformed with the plasmid pGEX-4T-3/PWP1 and a single colony was inoculated into 10 ml of LB medium containing ampicillin [100 µg/ml] and grown overnight at 37°C. This culture was diluted into 400 ml of LB with ampicillin and grown to $A_{595} = 0.5$. IPTG [0.4 mM] was added in order to induce the expression of the GST-fusion protein. Then, the bacteria were spun down at 5000 rpm in a J14 rotor for 15 minutes and resuspended in 10 ml of buffer A (50 mM Tris-HCl pH=7.5, 100 mM NaCl, 1 mM EDTA and protease inhibitor cocktail complete (Roche)). The cells were lysed by sonication for 20 seconds x 4 and ultracentrifuged at 39.000 rpm in a 70 TI rotor at 4°C for 1 hour. In order to purify soluble GST fusion protein after cell lysis the supernatant was incubated with 100-200 µl of 50% glutathione-agarose beads, resuspended in buffer A, and rotated at 4°C for 15 minutes and then spun down and washed 5 times in buffer A. After the final wash, the beads were resuspended in 2x SDS electrophoresis sample buffer and

immediately heated at 98°C for 5 minutes. To examine the insoluble fraction after ultracentrifugation the pellet was resuspended in 1 ml of 2x SDS sample buffer and heated at 98°C for 5 minutes. 20 µl of each fraction was loaded on a 10% SDS polyacrylamide gel in order to test the overexpression of the GST fusion protein. The fraction containing the GST fusion protein was purified from a preparative 10% SDS-PAGE gel, containing a single long well for sample loading. Electrophoresis was performed at a constant current of 25 mA. A strip from the gel was cut and stained with coomassie blue in order to locate the band of the overexpressed GST fusion protein. The strip was aligned with the gel and the area containing the protein of interest was cut out. This gel strip with a few drops of PBS was passed sequentially through syringe needle (10, 21 and 23 guage). The sample was sent to Biological Resources, ICRF Clare Hall Laboratories, for rabbit immunization.

Peptide conjugation for antibody preparation

Two peptides, PWP1-NT and PWP1-CT, corresponding to the amino- and carboxy-terminus, respectively, of PWP1 amino acid sequence were synthesised by the Biological Resources Department - ICRF. Both peptides were then conjugated with Keyhole Limpet Haemocyanin (KLH) (Calbiochem), as carrier, and then each peptide and carrier were weighed and the same amount of each was dissolved in 0.1 M NaHCO₃ at a carrier concentration of 2 mg/ml (G.Evan, personal communication). A fresh vial of 25% glutaraldehyde (Sigma) was thawed and added to the peptide/carrier mix to a final concentration of 0.05% and left mixing at room temperature overnight. The solution was adjusted to pH 7.0-8.0 using NaOH. The conjugated peptide was then dialysed against dH₂O for about 12 hours and

lyophilised. The samples were then sent to the Biological Resources, ICRF Clare Hall Laboratories, for rabbit immunization.

3.0 FISSION YEAST METHODS

Cell growth

Fission yeast manipulations were carried out as previously described (Moreno et al. 1991; Norbury and Moreno 1997). Fission yeast cells were maintained on YE5S agar (5 mg/ml yeast extract (Difco), 30 mg/ml glucose, 20 mg/ml agar (Difco, Bacto) and 225 µg/ml each of leucine, uracil, adenine, histidine and lysine hydrochloride). For selective maintenance of plasmids, the cells were grown on EMM2 agar (potassium hydrogen phthalate 14.7 mM, Na₂HPO₄ 15.5 mM, NH₄Cl 93.5 mM, glucose 20 mg/ml, MgCl₂ 5.2 mM, CaCl₂ 0.1 M, KCl 13.4 mM, Na₂SO₄ 0.28 mM, H₃BO₃ 8.1 µM, MnSO₄ 2.37 µM, ZnSO₄ 1.39 µM, FeCl₃ 0.74 µM, MoO₃ 0.25 µM, KI 0.6 µM, CuSO₄ 0.16 µM, citric acid 4.76 µM, nicotinic acid 81.2 µM, inositol 55.5 µM, biotin 40.8 µM, panthothenic acid 4.2 µM and agar 20 mg/ml (Difco Bacto)) supplemented with the appropriate amino acids at 225 µg/ml. Liquid cultures were grown in YE5S or EMM2 medium. Strains were stored in YE5S with 25% glycerol at -80°C.

Measurement of cell concentration

The cell concentration in liquid cultures was estimated by measuring absorbance at 595 nm, with an absorbance of 1.0 being equivalent to 2×10^7 cells/ml.

DNA transformation

S.pombe cells were transformed by electroporation.

Cells in mid-exponential growth in 50 ml EMM2 were harvested by centrifugation, washed in chilled sterile dH₂O and then in chilled sterile 1 M sorbitol. The cells were resuspended in 250 µl of 1 M sorbitol and 50 µl aliquots were mixed with up to 1 µl of transforming DNA, incubated on ice for 5 minutes and then electroporated (by a Bio-Rad gene Pulser set to 1.5 kV, 25 µF and 200Ω). 50 µl of EMM2 was added immediately to the cells which were then plated onto EMM2 agar with the appropriate supplements for plasmid selection. Plates were incubated at 30°C for 2-4 days until colony formation.

Expression of human Pwp1

PWP1 cDNA was cloned into pREP3X vector (Forsburg 1993), in which expression of the gene is under control of the *nmt1* thiamine-repressible promoter (Maundrell 1993). The resulting plasmid was transformed into *S.pombe leu1-32* by electroporation and transformants were selected on EMM2 agar plates without leucine. The plates also contained 10 µg/ml of thiamine to repress expression of the gene from the *nmt1* promoter. A single colony was subsequently inoculated into 10 ml of EMM2 medium containing thiamine and grown overnight at 30°C. Thiamine was removed by four washes with the same medium and the cells were grown for a further 24 hours at 30°C before analysis.

Protein extraction for immunoblot analysis

Whole cell lysates were prepared from 50 ml cultures in mid-exponential growth. The cells were harvested by centrifugation, washed once in dH₂O, lysed in 1 ml of 2x SDS sample buffer and heated immediately at 98°C for 5 minutes.

Drug resistance assay

EMM2 agar plates containing a series of drug concentrations of caffeine were prepared using a [100 mM] stock. MBC was added directly to boiling agar to produce 20 µg/ml stock, which was further diluted with agar to produce the necessary MBC concentration. Fission yeast cells in mid-exponential growth were counted using an automatic cell counter (Sysmex) and 1000 cells from each culture were spotted onto the drug-containing plates and immediately streaked with a sterile loop. Plates were incubated at 30°C for 3-4 days to allow colony formation.

4.0 CELL CULTURE

Cell lines and media

Monkey COS-7, human HeLa cervical carcinoma and U2-OS osteosarcoma cells were grown in D-MEM medium (glutamine 4 mM, streptomycin sulphate 100 mg/l, penicillin 100.000 units/l, antimycotic (Butyl-p-hydroxybenzoate) 0.2 mg/l) supplemented with 10% fetal bovine serum (FBS) (Bioclear) or 10% tetracycline-free fetal bovine serum (Clontech) for Poh1 inducible expression in U2-OS cells.

HTR9 cells are derived from the human HT1080 fibrosarcoma cell line and were maintained in culture in RPMI 2% glucose medium (glutamine 4 mM, streptomycin 100 mg/l, penicillin 100.000 units/l) supplemented with 10% fetal bovine serum (FBS) (Bioclear) and 150 µg/ml of hygromycin B for the stable selection of transfectants.

Transfections: FuGENE™ 6

Cells were transfected in 9 or 3.5 cm plates at 40-60 % confluence using FuGENE™ 6 reagent (Roche), according to the manufacturer's instructions.

3 μ l of FuGENETM 6 reagent for each μ g of DNA were mixed in 100 μ l final volume serum free medium and incubated for 5 minutes at room temperature in sterile conditions. The DNA, in a volume of up to 10 μ l, was dispensed into another sterile tube. 100 μ l of the FuGENETM 6 serum free medium mixture was then added to the DNA solution and left for 15 minutes at room temperature. The resulting mixture was added to 4.5 or 2 ml of complete medium (containing serum) for 9 and 3.5 cm plates, respectively. The medium containing the FuGENETM 6 plus DNA mixture was added to the cells and incubated for 6-8 hr or overnight.

At the end of incubation time, fresh medium (5 ml and 2 ml for 9 and 3.5 cm plates, respectively) was added to the cells, which were left in culture for further 24-48 hours

CD2 selection by magnetic beads

a) Preparation of anti-CD2-coated immunomagnetic beads

Under sterile conditions, 15 mg (0.5 ml) of rat anti-mouse IgG2a Dynabeads (DynaM-450) were prepared from the stock of 30 mg/ml (4 x 10⁸ beads/ml). The beads were washed three times in 4-5 ml of sterile-filtered PBS containing 0.1% bovine serum albumin (BSA), which was aspirated through a sterile Pasteur pipette after separation of the magnetic beads using a Dynal magnetic separator. The beads were then resuspended in 0.5 ml of PBS/0.1%BSA and incubated with a mouse monoclonal anti-CD2 antibody (OX-34, Serotec) at the concentration of 2 μ g per mg of beads for 30 minutes at 4°C on a rotating wheel.

The beads were washed three times with PBS/0.1% BSA and re-suspended in 0.5 ml of PBS/0.1% BSA to give a final concentration of 30 mg/ml.

- **CD2 immunomagnetic beads selection**

24 hours after transfection by FuGENE 6, cells were washed once with sterile PBS and harvested with PBS containing 2.5 mM EDTA. The cells were collected by centrifugation at 1000 rpm (MSE Mistral 2000) for 5 minutes, resuspended in 1 ml of D-MEM complete medium and incubated with 10-15 µl of the prepared anti-rat CD2 bead suspension at room temperature, giving a ratio of approximately 4 beads per cell. The beads/cells suspension was incubated at room temperature for 15 minutes with gentle agitation.

Bead-coated cells were separated using a Dynal magnetic separator and washed three times with sterile PBS followed each time by magnetic separation. The CD2⁺positive cells were resuspended in 1 ml of D-MEM complete medium and the number of cells was determined using a haemocytometer.

The positive cells were plated in complete medium for stable selection using the selective antibiotic, lysed for western blotting analysis or plated for drug sensitivity experiments.

Generation of stable transfectants

a) Polyclonal pool

- **COS-7:** the pcDNA3 or pcDNA3.1+ vectors expressing the gene of interest was co-transfected with pOPRSV/CD2 plasmid at a ratio of 4:1, using FuGENETM 6 reagent. 24 hours later, the CD2⁺ cells were selected as described above and plated, and 12 or 24 hours later fresh medium, containing 1 mg/ml G-418, was added. The cells were G-418 selected for about two weeks.
- **U2-OS:** the cells were co-transfected with pUHD10-MCS/HA-POH1 and pcDNA3.1+, to provide the G-418 resistance gene, as described for COS-7 cells.

24 after transfection, the cells were split to a low cell density and selected for about two weeks with G-418 [1 mg/ml].

b) Stable clones

- COS-7 stable clones expressing *HA-POH1* or *HA-PWPI* were derived from stable polyclonal pools, as follows: 1000, 500 and 100 cells were plated in G-418-containing medium. After 2 weeks, well-isolated colonies were picked using a Gilson P200 pipette and expanded. Protein expression was detected by immunoblot analysis.

- All the other stable clones were obtained as follows: 24 hours after transfection, the cells from each plate were harvested and divided between three plates, to allow individual colony formation. The selective antibiotic was added and the cells were allowed to grow for about two weeks, until colonies appeared.

Flow cytometry

Cells were trypsinised and collected by centrifugation (1.000 rpm for 5 minutes in MSE Mistral 2000), washed once in PBS and fixed in 1 ml of 70% ice-cold ethanol, added while vortexing, and incubated on ice for 30 minutes. Cells were then collected by centrifugation, washed three times with PBS and resuspended in 1 ml of PBS, containing propidium iodide (Sigma, 40 µg/ml) and RNase A (Sigma 100 µg/ml).

Red fluorescence was measured by flow cytometry (FACScan, Becton Dickinson) for 10000 cells per sample. Data analysis of the sample was performed using CellQuest software.

Annexin V binding assay

Annexin V binding assays were performed using the Annexine V - FITC detection kit (Pharmingen, Becton Dickinson), according to the manufacturer's guidelines. Cells were harvested in PBS/2.5 mM EDTA and washed twice on ice-cold PBS. 10^5 cells were resuspended in 100 μ l of 1x binding buffer. The cells were then incubated with 5 μ l of Annexin V - FITC and 2 μ l of Propidium Iodide (PI) for 15 minutes at room temperature in the dark. As staining controls, unstained cells, cells stained with PI alone and Annexin V alone were prepared. 400 μ l of 1x binding buffer was then added and the cells were analysed by flow cytometry (FACScan Becton Dickinson) within 1 hour.

Immunofluorescence

a) Indirect immunofluorescence

Cells were plated onto sterile glass coverslips and grown in 2 ml of medium in 6-multiwell plates the day before immunostaining. After washing in PBS, the cells were fixed on ice-cold 4% EM grade p-formaldehyde in PBS containing 250 mM Hepes pH=7.4 for 20 minutes at 4°C with agitation. The cells were then washed three times with PBS and permeabilized with cold PBS/0.5% Triton X-100 for 20 minutes at 4°C with agitation. The cells were then washed three times with PBS and incubated with blocking solution (PBS/0.1% Tween-20 + 10% FBS) for 0.5-1 hour at 37°C. The primary antibody (mouse monoclonal anti-HA antibody supplier) was diluted in blocking solution and the cells were incubated for 1 hour at 37°C. After three further washes in PBS/0.1% Tween-20, the cells were incubated with anti-mouse FITC-conjugated secondary antibody for 30 minutes, followed by three washes in PBS/0.1% Tween-20, one in PBS containing Hoechst 33258 [1 μ g/ml] and

a final wash in distilled water. The glass coverslips were dried and mounted (90% glycerol, 50 mM tris pH 8.8 and para-phenylenediamine 1 mg/ml) on glass slides. Immunofluorescence was assessed using an Axioskop microscope (Carl Zeiss, Oberkochen) through oil immersion objectives.

b) Localization of EGFP fusion proteins

Localization of EGFP-Pwp1 and EGFP-Poh1 fusion proteins was performed using the mammalian expression vector pEGFP, which expresses the gene of interest fused at the C-terminus of the enhanced green fluorescence protein (*EGFP*) sequence. HeLa or COS-7 cells, previously seeded onto sterile coverslips in 6-multiwell plates, were transiently transfected with the vector expressing the gene of interest or the empty vector. After 24-48 hours, the cells were washed once with PBS and fixed on ice-cold 4% EM grade p-formaldehyde in PBS/250 mM Hepes pH 7.4 solution for 20 minutes at 4°C with agitation. The cells were washed with PBS and then with PBS containing Hoechst 33258 [1 µg/ml] for 2-5 minutes, with final wash in distilled water. The glass coverslips were dried in a sterile hood and mounted in 90% glycerol, 50 mM Tris pH 8.8 and para-phenylenediamine (1 mg/ml) on glass slides. The expression of recombinant protein or EGFP alone was assessed using an Axioskop microscope (Carl Zeiss, Oberkochen) through oil immersion objectives.

Dual Luciferase Reporter Assay

2x10⁵ COS-7 cells were plated in 3.5 cm plates and 24 hours later the cells were transiently co-transfected using FuGENE™ 6 with pColLuc3 (kindly provided by Dr. J.Tavare) and pRL-TK-Renilla (Promega), at a ratio of 15:1. 24 hours after transfection, the cells were harvested, plated in triplicate in 6-multiwell plates and grown for further 24 hours before the assay.

The dual luciferase reporter assay kit (Promega) was used, according to manufacturer's instructions. The cells were lysed using 0.5 ml of PBL buffer 1x for 15 minutes with shaking. The lysed cells were collected in 1.5 ml eppendorf tube and spun down for 1 minute at maximum speed in an eppendorf centrifuge, in order to eliminate cell debris.

Both firefly and *Renilla* luciferase activities were measured using a luminometer (TD-20/20 Turner designs) with a 2 second pre-measurement delay followed by a 10 second measurement period for each reporter assay. For each sample 50 µl of LARII solution was predispensed into a luminometer tube, mixed by pipetting with 10 µl of cell lysate and emitted light measured using a luminometer. For *Renilla* luciferase activity, 50 µl of Stop & Glo solution was added to the same tube immediately after measuring the firefly activity, the tube was vortexed and re-read immediately.

The results were expressed as the ratio between the firefly and the *Renilla* activities (relative luciferase activity, RLA).

5.0 DRUG SENSITIVITY TESTS

a) Clonogenic survival assay

This assay was used in order to test the capacity of a cell population to form colonies, after exposure to a cytotoxic agent. 3000-4000 cells transiently or stably expressing the gene of interest or containing the empty vector were plated in 9 cm dishes. 24 hours later, the cells were exposed to different concentrations of a drug or no drug (as a negative control) for 24 hours; then, they were washed once in PBS 1x and incubated with fresh complete medium for 10-14 days. When colonies appeared, the medium was decanted and the colonies were fixed with 3 ml of a fixing solution (75% methanol + 25% acetic acid) for about 30 minutes, followed by staining with 3

ml of crystal violet (0.4 mg/ml) (1 hr to overnight). The plates were washed with tap water to remove excess of crystal violet and allowed to dry at room temperature. The colonies were then counted and the capacity to form colonies was expressed as the percentage survival in comparison with the control (cells with no drug).

b) MTS cytotoxicity assay

The MTS assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. 1000-2000 cells were plated in triplicate in a 96-multiwell plate (in 100 μ l final volume) and, after 24 hours, serial dilutions of a drug were added in 100 μ l final volume. The cells were incubated with the drug or no drug, as a negative control, for 72 hours at 37°C.

The MTS reaction was performed according to manufacturer's instructions. Briefly, in the dark and in sterile condition 20 μ l of MTS/PMS solution (100 μ l of PMS diluted in 2 ml of MTS) were added to each well, all containing the same volume, and incubated for 2 hours at 37°C. Cell viability was assessed by measuring A_{490} using microplate reader (Titertek Multiskan PLUS).

6.0 PROTEIN METHODS

SDS-PAGE

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing and reducing conditions, using the discontinuous buffer system described by Laemmli.

The resolving gel (10-12% polyacrylamide) was prepared in 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS. The stacking gel (4% polyacrylamide) was prepared in a buffer containing 125 mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS. All SDS polyacrylamide gels were assembled using the BIO-RAD Miniprotean vertical polyacrylamide gel

apparatus. Samples were heated for 3-5 minutes at 98°C in 2x SDS sample buffer (60 mM Tris-HCl pH= 6.8, 1% SDS, 5% glycerol, 0.001% bromophenol blue and 1% 2- β -mercaptoethanol) before loading on the gel.

Electrophoresis was performed at 120 V in a buffer containing 190 mM Glycine, 25 mM Tris and 0.1% SDS.

Whole cell extracts for immunoblot analysis

Growing cells were trypsinized and washed once with PBS. The cells were lysed in 2x SDS sample buffer (1 ml for a 9 cm confluent dish) and heated immediately at 98°C for 5 minutes before storage at -20°C or loading on the gel.

Immunoblot analysis

After electrophoretic separation, proteins were transferred from the gel on to a Hybond ECL nitrocellulose membrane (Amersham) by electroblotting, using the BIO-RAD Trans-Blot apparatus. The electroblotting was performed at 50 Volts for 2 hours at 4°C or 20 Volts for 16 hours at 4°C in a transfer buffer containing 20% methanol, 50 mM Tris and 380 mM glycine. The membrane was incubated in a blocking solution (PBS containing 0.3% Tween-20 and 5% of Marvel dried milk powder) for 1 hour or overnight with shaking. The primary antibody was diluted in the blocking solution and the membrane was incubated with the diluted antibody for 1 hour at room temperature with shaking. The membrane was washed four times in PBS/0.3% Tween-20 for about 1 hour and then incubated with an HRP-conjugated secondary antibody, diluted in blocking solution, for 30-45 minutes at room temperature. The membrane was washed 4 times with PBS/0.3% Tween-20 and once

with TBS 1x before detection by ECL method.

Enhanced Chemiluminescence (ECL) detection

Oxidation of luminol results when horseradish peroxidase (HRP), which is conjugated to a secondary antibody, reacts with luminol and chemical enhancers. This reaction produces light emission, which is detectable using X-ray film (Kodak). Equal volumes of solution I and II (ECL, Amersham) were mixed and the membrane was incubated with this mix for 1 minute. The membrane was drained, covered with Saran-wrap and then exposed to X-ray film for the required length of time.

Membrane reprobing

To probe the membrane with second antibody, recognising a protein of a similar size to that detected previously, it is necessary to completely remove the antigen-bound primary and secondary antibodies.

The membrane was incubated with stripping buffer (100 mM β -mercaptoethanol, 2% sodium dodecyl sulphate and 62.5 mM Tris-HCl pH 6.7) at 60°C for 30 minutes. The membrane was then washed twice with an excess of PBS/0.3% Tween-20 and then used again for a new immunoblot analysis.

Immunoprecipitation (IP)

c-Jun - Immunoprecipitation was performed as described by Norbury *et al.* (Norbury *et al.* 1991). Cell lysates were prepared in lysis buffer containing 20 mM Tris pH 7.4, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Complete, Roche) and incubated on ice for 30 minutes. Then, the lysates were spun down at maximum speed in an eppendorf centrifuge for 15 minutes at 4°C and the

supernatant was incubated with primary antibody for 1 hour on ice. 0.1 volume of a 50% suspension of protein A Sepharose (Sigma) was added and incubated for 2 hours in a rotating wheel at 4°C. The immunocomplexes were washed 5 times using lysis buffer. For immunoblot analysis an equal volume of immunoprecipitate and 2x SDS sample buffer was mixed, heated at 98°C for 5 minutes and loaded onto a 10-12% SDS-PAGE gel.

MCP-21 - Immunoprecipitation was carried out as described by Hayes *et al.* (Hayes and Wolf 1990). 10 million cells were resuspended in 5 volumes of lysis buffer (50 mM tris-HCl, 17% glycerol pH=7.5, 5 mM ATP and EDTA-free protease inhibitor cocktail (Complete, Roche)) and sonicated on ice for 10-20 seconds x 4. The lysates were spun down at maximum speed in an eppendorf centrifuge for 10 minutes at 4 °C and the supernatant was collected and incubated with the mouse monoclonal MCP-21 antibody for 1 hour on ice. The antigen-antibody complex was pulled down with 0.1 volume of a 50% protein G sepharose suspension for 4-6 hours on a rotating wheel at 4 °C. Then the complexes were washed 4-5 times in the following buffer: 20 mM Tris-HCl pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 1 mM MgCl₂, 0.5% Nonidet P-40, 0.1% SDS, 17% Glycerol, 5 mM ATP and EDTA-free protease inhibitor cocktail (Complete, Roche).

The immunoprecipitates were resuspended in 2x SDS sample buffer, heated at 98 °C for 5 minutes and loaded onto a 10% SDS-PAGE gel before being processed by immunoblotting for HA and C3 detection.

Electrophoretic mobility shift assay (EMSA)

a) Whole cell extracts

80-90% confluent cells from a 9 cm plate were washed once with PBS and then collected using a scraper in about 1 ml of PBS. The cells were spun down for 1 minute at maximum speed in an eppendorf centrifuge at 4°C, resuspended in about 60 µl of ice-cold lysis buffer (20 mM Hepes pH 7.9, 1 mM EDTA, 0.4 M NaCl, 25% Glycerol, 2.5 mM DTT, 5 mM NaF, 0.5 mM Na₃VO₄, 0.1% NP-40 and 1 tablet of EDTA-free protease inhibitor cocktail (Complete, Roche)) and incubated on ice for 30 minutes. The cells were spun at maximum speed in an eppendorf centrifuge for 15 minutes at 4°C and the supernatant was collected.

Protein quantification was performed using the BCA* Protein Assay Reagent A (Pierce).

b) Nuclear Extracts

Nuclear extraction was performed as described by Dignam *et al.* (Dignam et al. 1983), with some modifications. Cells were trypsinized, washed in 10 ml of ice-cold PBS and then in 1 ml ice-cold PBS. The cells were transferred to a 1.5 ml eppendorf tube and spun down for 3 minutes at 3000 rpm in an eppendorf centrifuge. 3 cell pellet volumes of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM NaF, 0.5 mM Na₃VO₄, 5 mM DTT and EDTA-free protease inhibitor cocktail (Complete, Roche)) was added to the cells and incubated on ice for 1 hour. The cells were then lysed by 20-40 strokes with a dounce homogeniser. The nuclei were spun at 13000 rpm in an eppendorf centrifuge for 10 minute at 4°C and the nuclear pellet was resuspended in buffer A (same volume as before) and 3 volumes of buffer B (20 mM HEPES pH 7.9, 20% Glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 0.5mM NaF, 0.5 mM Na₃VO₄, 5 mM DTT and 1 tablet of protease inhibitors

cocktail EDTA-free) and incubated on ice for 30 minutes. The nuclei were spun at 13000 rpm in an eppendorf centrifuge for 30 minutes at 4°C and aliquots of the supernatant were frozen in dry ice and stored at -70°C. Protein quantification was performed using the BIO-RAD protein assay (BIO-RAD).

c) End-labelling of double strand oligonucleotide

5 ng of AP-1 consensus double strand oligonucleotide (5'-CGCTTGATGACTCAGCCGGAA-3') was incubated in 10 µl reaction containing 1x T4 polynucleotide kinase buffer, 2.5 µCi of [³²P]-γATP and 1 µl of T4 polynucleotide kinase for 1 hour at room temperature. The labelled oligonucleotide was purified from the unincorporated radioactivity using the mini quick spin DNA columns (Roche) according to the manufacturer's instructions.

d) Electrophoretic Mobility Shift Assay (band-shift assay)

For gel shift analysis, whole cell or nuclear extracts were used. Binding reactions were performed at room temperature for 30 minutes with 8-10 µg of whole cell extracts or 5 µg of nuclear extracts. The extracts were mixed with 0.5 ng of [³²P]-γATP labelled AP-1 consensus probe (5'-CGCTTGATGACTCAGCCGGAA-3') [Santa Cruz Biotechnology, Inc] and 1 µg of poly (dI-dC) (in order to inhibit non specific binding of labelled probe to proteins) in 20 µl final volume of 1x binding buffer (10 mM Tris-HCl (pH=7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% of Glycerol). For competition experiments, an excess (50-fold) of cold double strand AP-1 consensus or AP-1 mutant (5'-CGCTTGATGACTGTGCCGGAA-3') oligonucleotides (Santa Cruz Biotechnology, Inc) was added to the binding reaction for 20 minutes before the labelled probe at room temperature.

DNA-protein complexes were resolved by electrophoresis under non-denaturing conditions using a 4% polyacrylamide gel containing 50 mM Tris, 0.38 M glycine

and 2 mM EDTA. The gel was subsequently dried for 2 hr and exposed to X-ray film overnight at -70°C with an intensifying screen or visualised using a storm phosphorimager system (Molecular Dynamics).

***In vitro* degradation assay**

In vitro degradation assays were performed as described by Brandeis et al. (Brandeis and Hunt 1996). Cells were washed twice in PBS and twice on ice-cold hypotonic buffer (20 mM Hepes pH 7.5, 5 mM KCl, 1.5 mM MgCl_2 , 1 μM dithiothreitol (DTT)). The buffer was completely aspirated and the cells were scraped off and disrupted with several strokes of a Dounce homogeniser. The cells were then spun down at maximum speed in an eppendorf centrifuge at 4°C for 15 minutes to remove cell debris and the supernatant was aliquoted and snap frozen immediately in liquid nitrogen and then stored at -80°C . Protein amount was measured using the BIO-RAD protein assay (Bio-Rad).

Degradation assays were performed in 10-15 μl final volume containing an equal amount of protein from each cell lysate, supplemented with an energy-regenerating system (25 mM phosphocreatine, 10 $\mu\text{g/ml}$ creatine kinase and 1 mM ATP) and 0.5-1 μl of the [^{35}S]-methionine radiolabelled substrates, human c-Jun₁₋₂₈₇, which lacks the leucine zipper domain, and c-Jun Δ_{3-122} , which lacks the δ domain (both synthesised using plasmids kindly provided by Dr. D.Kardassis). Reactions were incubated at 30°C and each reaction was stopped at the time points 0, 20, 40, 70, 100 and 160 minutes by adding 2x SDS sample buffer and immediately heated for 5 minutes at 98°C . The samples were loaded onto a 12% SDS-PAGE gel for protein separation. The gel was dried and exposed to a X-ray film using an intensifying screen at -80°C for the necessary length of time. The substrates were [^{35}S]-labelled *in*

vitro transcribed and translated in nuclease-free reticulocyte lysates using a TNT T7 Quick Coupled Kit (Promega), according to the manufacturer's instructions.

Purification of His₆-tagged c-Jun

U2-OS cells were plated in 9 cm dishes and transiently co-transfected with 2 µg of a vector expressing a His₆-tagged human c-Jun (pMT35/His₆-c-Jun) and 2 µg of a vector expressing the HA-tagged Ubiquitin (pcDNA/HA-Ub). The cells were mock or doxycyclin [1.5-2 µg/ml] treated for 36 hours before nickel chelate affinity purification of the protein, which was performed as described by Treier *et al.* (Treier et al. 1994).

The cells were lysed in 2 ml/plate of 6 M guanidinium-HCl, 0.1 M sodium phosphate pH 8.0 and 5 mM imidazole and sonicated in order to reduce the viscosity. Cleared lysate or buffer alone (4 ml), as negative control, was incubated with 400 µl of NiSO₄-charged His-bind resin (Novagen) (50% slurry) for 4 hours at room temperature on a rotating wheel. The slurry was then applied to a 0.8 x 4 cm chromatography column (Bio-Rad), which was successively washed with the following solutions: 1 ml of 6 M guanidinium-HCl, 0.1 M sodium phosphate pH 8.0; 2 ml of 6 M guanidinium-HCl, 0.1 M sodium phosphate pH 5.8; 1 ml of 6 M guanidinium-HCl, 0.1 M sodium phosphate pH 8.0; 2 ml of 1 ml of 6 M guanidinium-HCl/0.1 M sodium phosphate pH 8.0 : protein buffer [1:1]; 2 ml of 1 ml of 6 M guanidinium-HCl/0.1 M sodium phosphate pH 8.0 : protein buffer [1:3]; 2 ml of protein buffer; 1 ml of protein buffer containing 10 mM imidazole. Protein elution was carried out with 1 ml of protein buffer containing 200 mM imidazole. The protein was TCA (100%) precipitated for 30 minutes on ice, followed by centrifugation at maximum speed in an eppendorf centrifuge at 4°C. The pellet was

resuspended in 40 µl of 2x SDS sample buffer plus 1 µl of 1 N NaOH, in order to adjust the low pH of the sample, and heated at 98°C for 5 minutes. The samples were loaded onto a 10-12 % SDS-PAGE gel and c-Jun and the ubiquitinated level of c-Jun were detected using rabbit polyclonal anti-c-Jun and HRP-conjugated monoclonal anti-HA antibodies, respectively.

The His-bind resin was prepared as follows: 1.2 ml of 50% slurry was washed once with PBS and then incubated with 1 ml of 0.4 M NiSO₄ for 15-30 minutes on a rotating wheel at room temperature. The slurry was washed in lysis buffer and resuspended in 1.2 ml.

Sucrose density gradient centrifugation

Growing cells from 4 flasks (175 cm) were harvested and washed once with PBS before being resuspended in 300 µl of lysis buffer (10 mM tris pH 7.5, 100 mM NaCl, 30 mM MgCl₂, 50 µl/ml cycloheximide and 5 mM ATP). The cells were successively passed through different size needles until complete lysis. The lysates were then centrifuged at 40.000 rpm (Beckman SW41 rotor) for 1 hour and 45 minutes. The lysates were applied to a 13.5 ml 7-37% sucrose gradient prepared in 50 mM tris acetate, 50 mM NH₄Cl, 12 mM MgCl₂ and 5 mM ATP. Sucrose gradients were made up stepwise using 37, 27, 17 and 7% sucrose, starting with 37%. Each step was frozen before addition of the next and the gradients were stored at -20°C. After the lysated cell were centrifugated, 0.5 ml fractions were collected (Pharmacia FRAC-100) by displacement of the gradient from below using perfluorodecalin (Fluorochem, Old Glossop, UK). 20 µl aliquots of each fraction were subjected to analysis by SDS-PAGE and immunoblotting.

7.0 YEAST TWO HYBRID SCREEN

Plasmids

Budding yeast *S.cerevisiae* EGY48 strain was used in the two-hybrid interaction screen. This strain contains two *lexA* operator-responsive reporters: one is a chromosomally integrated copy of the *LEU2* gene and the second is a plasmid bearing the *GAL1* promoter-*LacZ* gene, which causes the yeast cells to turn blue on agar containing X-gal. To generate the bait protein, the human *PWP1* cDNA was cloned into pEG202 generating a fusion with the heterologous DNA binding protein LexA. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus and it should not possess intrinsic ability to strongly activate transcription. The pEG202/PWP1 was transformed by the lithium acetate method into the EGY48. The transformed cells were selected by plating on complete agar -Ura -His- dropout plates, with glucose as carbon source, and incubated at 30°C for two to three days. In order to check if the LexA-PWP1 fusion protein was able to activate transcription without interacting partners, the *PWP1*-containing cells were transformed with the pJG4-5 empty vector (the vector where the human cDNA library is expressed from) by lithium acetate. The transformed cells were selected in Ura -His -Trp complete agar plates (containing glucose) and allowed to grow for 2-3 days. Then, the β -galactosidase assay was performed: 6-10 colonies were streaked in an YPD agar plate and allowed to grow overnight. Cells were replica plated onto - Ura -His -Trp -Leu dropout agar plates containing galactose/raffinose (gal/raff) (Ura, His, Trp, Leu (UHTL) 0.65 g/l, yeast nitrogen base 6.5 g/l, ammonium sulphate 5 g/l, galactose 20 g/l, raffinose 10 g/l and agar 20 g/ plus sodium phosphate 0,1 M pH=7.5) and X-gal 50 μ g/ml and incubated at 30°C overnight for 24 hours.

Lithium acetate transformation

Yeast cells were grown to mid-exponential phase in 10 ml culture of SD medium containing the appropriate amino acid supplements. The cells were harvested by centrifugation at 3000 rpm in an eppendorf centrifuge for 5 minutes at room temperature and washed once in water. The pellet was resuspended in 1 ml of lithium acetate/TE 1x (Li/TE) solution, centrifuged for 30 seconds at 8000 rpm and resuspended in 50 µl of Li/TE. 1-2 µl (about 1 µg for single vector and 5 µg for the cDNA library) of transforming DNA and 5 µl of salmon sperm DNA [10 mg/ml] and the mixture was incubated for 10 minutes at room temperature. 300 µl of Li/TE plus 40% PEG solution was added to the cells and incubated for 1 hour at 30°C. The cells were then heat shocked at 42°C for exactly 5 minutes and centrifuged immediately at 8000 rpm in an eppendorf centrifuge for 5 minutes, washed in dH₂O, resuspended in 1 ml of YPD medium and allowed to recover at 30°C for 1 hour. The cells were spun down and resuspended in 100 µl of SD medium and plated onto SD agar plates containing the appropriate amino acid supplement.

The interaction hunt

The interaction hunt was performed using the LexA-Pwp1 bait and a human cDNA library in pJG4-5.

Each cDNA sequence of the human HeLa cDNA library is fused to the GAL promoter in the pJG4-5 vector. After transformation of 2-5 µg of cDNA library by lithium acetate, yeast cells were first plated on SD (complete agar) -Ura -His -Trp dropout agar plates, containing glucose as carbon source, to select for library plasmids. After transformation the yeast cells were incubated at 30°C for 3-5 days, until colonies appeared. The cells were replica plated onto -Ura -His -Trp -Leu

(UHTL) dropout Gal/Raff agar plates, (UHTL 0.65 g/l, yeast nitrogen base 6.5 g/l, ammonium sulphate 5 g/l galactose 20 g/l, raffinose 10 g/l and agar 20 g/l plus 0,1 M sodium phosphate pH 7.5 and X-Gal 50 µg/ml). Blue colonies found after incubation for 16 to 36 hours at 30°C were restreaked on complete medium plates to ensure that a single colony had been picked up, and then replica plated onto UHTL gal/raf X-gal plates to further check the positive interaction.

Rescue of cDNA library plasmids

Each positive blue colony was grown in 10 ml of complete medium (SD medium) - Trp overnight at 30°C. 1 ml of this yeast culture was spun down and the pellet was washed twice with dH₂O and resuspended in 100 µl of dH₂O. DNA was extracted by vortexing with 100 µl of phenol/chloroform and sterile glass beads. The mixture was mixed with a vortex for 10-15 minutes, in order to break the yeast wall, and then spun down and the aqueous phase was collected. The extraction was repeated and the genomic and plasmid DNAs were precipitated with 0.3 M sodium acetate and 70% ethanol. The DNA was resuspended in 20 µl of dH₂O and 1 µl was used to transform an *E.coli trp*-mutant strain by electro-transformation, in order to select the vector containing the cDNA. A single colony was inoculated in LB medium containing ampicillin [50 µg/ml], allowed to grow overnight at 37°C and the plasmid DNA was extracted by mini-prep. The cDNA sequence was checked using the oligonucleotides PJG45X and PJG45E (see A.1 - *Appendix*). In parallel, 1 µg was re-transformed into EGY48, containing the reporter vectors and LexA-Pwp1 bait, in order to further check the previously detected interaction.

Chapter 3

Further characterization of the 26S proteasome regulatory subunit Poh1

Overview

In chapters 3, 4, and 5 of this thesis I describe the further characterization of the regulatory proteasomal subunit Poh1 and the HA-Poh1-conferred pleiotropic drug resistance phenotype previously observed in mammalian cells.

The main aspects on which I focus are:

- The expression of *HA-POH1* using a constitutive and an inducible expression system in monkey COS-7 and human U2-OS cells, respectively
- Characterisation of the drug resistance phenotype using a wide range of drugs in both systems
- The sub-cellular localization of HA-Poh1
- The association of HA-Poh1 with the 26S proteasome complex
- The molecular mechanism by which HA-Poh1 causes pleiotropic drug resistance, including investigation of alterations in AP-1 transcription factors, the ubiquitinylation pathway and AP-1 stability

In this chapter I show that the stable expression of exogenous epitope-tagged HA-Poh1 (HA-Poh1) in mammalian cells is able to confer multi-drug resistance to a wide range of drugs. In addition, I also describe and discuss the cytoplasmic and nuclear localization of HA-Poh1 and its association with the 26S proteasome.

Introduction

POH1

POH1, for *pad* *one* *homologue* *1*, was first described in 1997 (Spataro et al. 1997) as the human homologue of the fission yeast multi-drug resistance gene *pad1*⁺ (Shimanuki et al. 1995). The primary sequence similarity between the human and the fission yeast proteins is 68% and Poh1 and Pad1 are also related, more distantly, to the c-Jun-activating-binding protein Jab1 and the human 26S proteasome regulatory subunit S12/p40 (Figure 3.1). High degree of conservation of *pad1*⁺/*POH1* during evolution indicates a fundamentally important role for the corresponding proteins. *POH1* mRNA is widely expressed in many human tissues with the highest level in the heart and the skeletal muscle (Spataro et al. 1997). A similar pattern was previously observed for the S12/p40 proteasome subunit.

The functional characterization of the budding yeast *pad1* homologue *MPR1* has also been reported (Rinaldi et al. 1998). Like its counterparts in fission yeast and human cells, the gene encodes a regulatory subunit of the 26S proteasome complex, named Mpr1/Rpn11 (Glickman et al. 1998b). The temperature sensitive mutant strain *mpr1-1* encodes a truncated protein that lacks the last 31 amino acids at the C-terminus and also shows pleiotropic phenotypes.

1. Cell growth defects which are complemented by the expression of the human *HA-POH1* gene
2. Overreplication of the mitochondrial and nuclear DNA
3. Accumulation of ubiquitinated proteins, which is also consistent with the data reported for other mutants of the 26S regulatory subunits.

```

Poh1  1  MDRLLRLLEGGMPLGLGQGPPTDAFAWDTAEDWV.....
Pad1  1  MESLORLLOGAR.MSTEMMGDOFLVDNSECWV.....
Jab1  1  ...MAASGESEMAOKTNELANMMOEAOISI DEIYKYDKKQOOEI LAAKPNTK
p40   1  .....M

Poh1  33  .....ISSLALLKMLKH....GRAGVPMEVMGLMLGFEVDVTVRV
Pad1  32  .....ISSLALLKMLRH....GRHGTPEMEVMGLMLGFEVDVTVRV
Jab1  48  DHYFKYCKISALALLKMVMH....ARSGGNLEVMGLMLGK.VDGETMIIL
p40   2  PELAVQKVVPHPVLVLLSYVDHFNRIYKVENQKRNVGVLLGSN.QKKVLDV
          *  **      *          *      *  *  **

Poh1  70  IDVFAMPOSCTGVSVEAUDPVFO..AKMLDMLKDTGRPEMVVGWYHSHPG
Pad1  69  YDVFAMPOSCTGVSVEAUDPVFO..KMMMDMLKDTGRPEMVVGWYHSHPG
Jab1  93  MDSFALPVEGTETRYNMDAAAYEYMAAYIEVANKDVGHLENALGWYHSHPG
p40   51  SNSEAVHFDDEDDKDDSVFLDHDVLENMYGMFKKVNARERIVGWYHT...
          **  *          *          *      ***
          ↓

Poh1 118  FGCNLSGV DINTQOSFEALSERAVAVVVD.....
Pad1 117  FGC LSSVDINTQOSFEOLT PRAVAVVVD.....
Jab1 143  YGCNLSGI DVSTQMLNQDFQEPFVAVVVD.....
p40   98  .GPKLHKNDIAINELMKRYCPNSVLVNI DVKPKDLGLPTEAVISVEEDQD
          *  *      *          *  *  *

Poh1 147  ....PIQSVK.GKVVIDAFRLINANMMVLGHEPRO.TTSNLGHINLKPSIQ
Pad1 146  ....PIQSVK.GKVVIDAFRLINPSTLMNGOEPRO.TTSNLGHINLKPSIQ
Jab1 172  ....ETRTL.SAGKVNLGAFRTYPKGYKPPDEGESEYOTIPLNKIEDFGVH
p40  147  DGTPTSKTTFEHVTSEIGAEAEVVEYENLLRDINDTIVGTLSQRITNQVH
          *                      *

Poh1 191  AL....IHGLNRHYVSTTINVRKNELECKMLLNHLHKKS.NMEGLTLQDYS
Pad1 190  AL....IHGLGRHYVSLRINVRKNTLEEEIMLLNLHKQP.NAKELLLENFN
Jab1 218  C.....KQYVALEVSVFNSSLDRKLELLLNWNV.NVNTLSSSSLL
p40  197  QLKGLNSKLLDIQSYLENVGTGNLPINHQIIVQLQDVFNLLPDVSLQEFV
          *          *          *

Poh1 236  EH...CKHNE SVUKEMLELAKNVNK..AVEEEDKMTPEOLAIKNVGRQDP
Pad1 235  SR...AEKNHASIDKMKSLSEDTTE..RWQNEVTLSPEELRIQVVGKQDP
Jab1 257  TN...ADYTTGQVFDLSEKLELSEA..QLGRGSFM...LGLTTHDRKSE
p40  247  KRFFYLKTN DQMVVVYLA SLIRSVVALHNLI MNKI ANRD AEKK EGQEMEES

Poh1 281  KRKLEEHVDVLMT..SNI VQCLAAMLDTVVFK.....
Pad1 280  KRKLDAAEVOKCID..NNISSMLACMLDSVAF.....
Jab1 298  DKLAMATRDSCKTTIEALHGLMSQVTKDKLENQINIS
p40  297  KKDRKEDEKEDKDKDKEKSDVKKEEKKEKK.....

```

Figure 3.1 – Poh1 protein sequence alignment with fission yeast Pad1 and human Jab1 and S12/p40

The residues present at identical positions in two or more proteins are highlighted in *black*; conservative substitutions in *grey*. The putatively ubiquitin hydrolase site in Poh1/Pad1 is marked with an arrow (▼). The 28 residues present at identical position in the four proteins are marked with an asterisk (*).

Mpr1 was the first proteasomal subunit described with a possible role in mitochondrial biogenesis (Rinaldi et al. 1998). Mpr1/Rpn11 was also recently reported to be required for the UV-induced activation of the leucine-zipper transcription factors Gcn4 (Stitzel et al. 2001).

POH1 in S.pombe

Poh1 is a fully functional homologue of fission yeast Pad1 (Spataro et al. 1997). When overexpressed from the thiamine-repressible promoter of the *nmt1* (*no message in thiamine*) gene, *POH1* was able to rescue the viability of a fission yeast haploid strain bearing a null mutation in the essential *pad1*⁺ gene (Δ *pad1*). When the promoter was repressed through the addition of thiamine into the medium, cells lost viability very rapidly and also showed some mitotic defects. Overexpression of HA-tagged Poh1 in fission yeast led to resistance to the protein kinase inhibitor staurosporine. In addition, as observed previously for the *S.pombe pad1*⁺ gene (Shimanuki et al. 1995), the drug resistance phenotype induced by HA-Poh1 overexpression was dependent on *pap1*⁺ (Spataro et al. 1997).

POH1 in mammalian cells

Transient overexpression in monkey COS-1 cells of the haemagglutinin (HA) epitope-tagged human *HA-POH1* gene, under the control of the CMV promoter in the pcDNA3 mammalian expression vector (pcDNA3/HA-POH1), led to resistance to several unrelated agents such as taxol, 7-hydroxystaurosporine (UCN-O1) and UV light when compared to the cells carrying the empty vector (pcDNA3) (Spataro et al. 1997). In contrast, no significant difference in cell survival was observed when the

cells were exposed to ionising radiation or to hydrogen peroxide. Thus, the transient overexpression of HA-Poh1 in mammalian cells induced a distinctive pattern of multi-drug resistance.

In addition, the HA-Poh1-conferred multi-drug resistance phenotype in COS-1 cells was not accompanied by elevated expression of P-gp (Spataro et al. 1997).

Poh1 has sequence similarity to the S12/p40 subunit of the 19S regulatory particle of the 26S proteasome complex (Spataro et al. 1997) (Figure 3.1) and was found to be a novel subunit of the 19S regulatory particle (Spataro et al. 1997). The Poh1 homologues in budding and fission yeasts were subsequently shown also to be 26S proteasome components. Later reports have also confirmed that Pad1/Poh1 are regulatory subunit of the proteasomal complex (Glickman et al. 1998b; Holzl et al. 2000). Most of the endogenous HA-Poh1 was associated with the 26S proteasome complex and very little was present as a free monomer or other relative low molecular weight forms. This is also the case for the majority of the previously characterised components of the 20S core complex.

The 26S proteasome complex

Protein degradation is a key regulator of many cellular processes and it is modulated by both spatial and temporal controls. In eukaryotic cells there are two major proteolytic pathways. The lysosomal pathway mainly degrades extracellular proteins that have entered the cell by endocytosis or pinocytosis; the major non-lysosomal pathway, the so-called ubiquitin-proteasome pathway, degrades intracellular proteins that have been previously tagged by the protein ubiquitin.

The 26S proteasome is a multi-protein complex that is implicated in the turnover of mitotic cyclins, oncoproteins, the tumor suppressor protein p53, cell surface receptors,

transcriptional regulators and mutated and damaged proteins (reviewed by Ciechanover 1994). The 26S proteasome was first isolated in 1986 (Hough et al. 1987) and it was recently confirmed that the complex derives from the association of the 20S proteasome and two 19S regulatory complexes, also called the PA700, at both ends of the 20S proteasome with a C_2 -symmetry (Figure 3.2C). Both assembly and disassembly of the 26S proteasome are energy-dependent. There is a dynamic equilibrium between the free 20S and 19S forms and the 26S proteasome complex (reviewed by Voges et al. 1999).

The 20S proteasome

The 20S proteasome, also referred to as the core particle (CP), was isolated in 1968 from human erythrocyte lysates as a cylinder-shaped structure. The structure was initially called ‘cylindrin’ but its function was unknown (Harris 1968). It is 700 kDa complex containing subunits in the 24-28 kDa range. Eukaryotic and prokaryotic 20S proteasomes are broadly similar but differ in complexity; each is composed of 28 subunits. The prokaryotic proteasome contains 14 copies of two distinct but related proteins α and β ($\alpha_7 \beta_7 \beta_7 \alpha_7$), whereas the eukaryotic proteasome consists of two copies each of 7 distinct α -type and 7 distinct β -type subunits ($\alpha_1-\alpha_7$)($\beta_1-\beta_7$)($\beta_1-\beta_7$)($\alpha_1-\alpha_7$). In both the prokaryotic and the eukaryotic complexes these proteins form a ring structure. Two juxtaposed rings of α -type subunits flanked on the top and bottom by a ring of β -type subunits form the barrel-shaped complex with a C_2 -symmetry (Figure 3.2A). As judged by electron microscopy the 20S proteasome is 15 nm in height and 11 nm in diameter. The central chamber formed by the 4 rings may trap the proteins until they are degraded to generate peptides with a

size ranging between 4-25 residues and an average length of 7-9 residues (reviewed by Voges et al. 1999).

The 19S regulatory particle

Given that access to the proteolytic compartment is restricted to unfolded proteins, other components associated with the 20S proteolytic structure would be expected to recognise and bind and unfold the target substrate.

The 19S particle is regulatory complex that can bind to each end of the cylindrical 20S proteasome (DeMartino et al. 1994) (Figure 3.2B). The 19S particle contains the ATPase subunits and acts through an energy-dependent mechanism. It has three main biochemical functions.

1. The recognition of the polyubiquitinated substrates (Deveraux et al. 1994)
2. An isopeptidase activity which is necessary for the recycling of the ubiquitin molecules from the substrate (Lam et al. 1997)
3. An anti-chaperone activity that unfolds the substrate and presents it to the 20S catalytic complex (Braun et al. 1999).

The 19S particle is dissociable into two subcomplexes, the base and the lid (Glickman et al. 1998a).

The base complex

The base complex contains all the six ATPase subunits (S7/Rpt1, S4/Rpt2, S6/Rpt3, S10b/Rpt4, S6/Rpt5 and S8/Rpt6) and the two largest subunits (S1/Rpn2 and S2/Rpn2) as well as S5a/Rpn10. Each of the six ATPase subunits contains the 230 amino acid AAA or CAD module that is a characteristic of the family of the AAA ATPases. The complex formed by the 20S proteasome and the base of the 19S

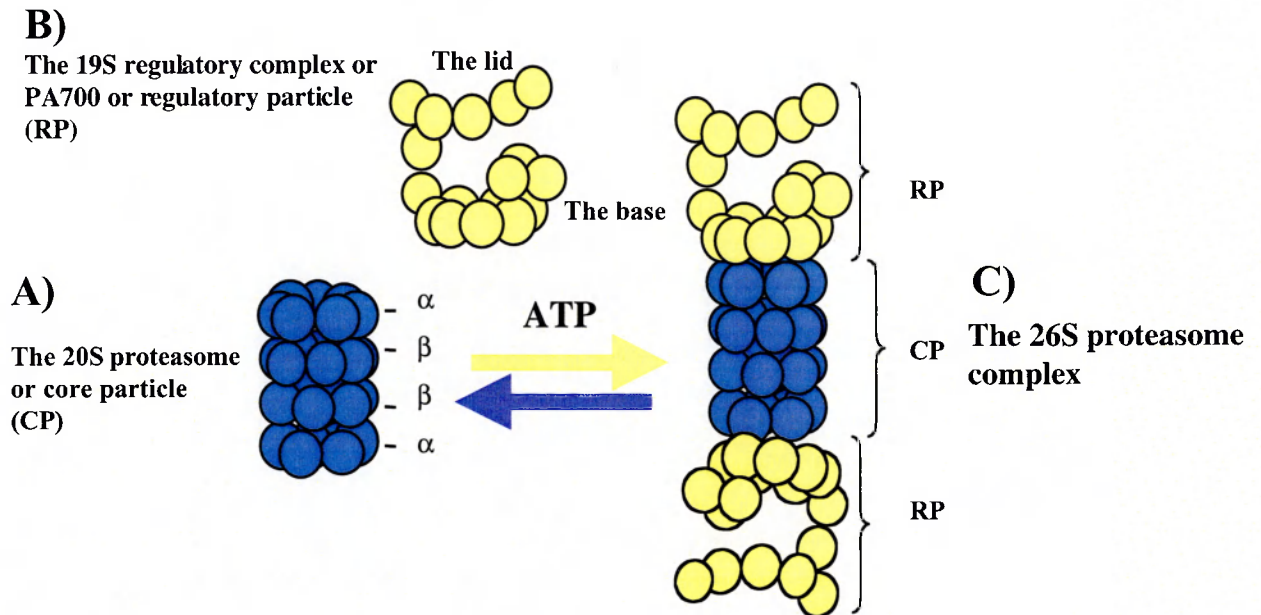


Figure 3.2 – The 26S proteasome complex: assembly of the core (20S) proteasome with the 19S regulatory complex

The 19S regulatory complex, also called the ATPase complex or the proteasome activator 700 (PA700), binds to both ends of the 20S proteasome with a C_2 -symmetry forming the 26S proteasome complex, which is now able to recognize and degrade ubiquitinated proteins.

regulatory complex is capable of ATP-dependent protein degradation but it cannot recognise and degrade ubiquitinated proteins (Voges et al. 1999).

It has been suggested that the six ATPases of the base assemble in a ring that forms the interface of the 19S particle with the 20S proteasome. Some studies carried out in *Xenopus*, *Thermoplasma* and *Drosophila* suggest that the six ATPases directly bind to the seven-member α -type subunit ring of the 20S proteasome. Several functions have been proposed for the role of the ATPases in the 19S regulatory particle (Voges et al. 1999).

- The hydrolysis of the ATP by the 19S complex is thought to promote the assembly of the 19S and the 20S complexes to form the 26S proteasome complex
- The ATPases might have a role in the gating of the translocation channel
- Substrate proteins are possibly bound to and unfolded by the ATPases
- The ATPases might assist in the translocation of the unfolded substrate proteins into the central chamber of the 20S proteasome

The lid complex

The lid complex is essential for the recognition and the degradation of the ubiquitinated substrates through the 26S proteasome. It is the distal component of the 19S regulatory particle and it contains 8 subunits (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11 (Pad1/Poh1) and Rpn12) (Voges et al. 1999).

Two structural domains, the PCI (Proteasome, COP9 and eIF3) and the MPN (Mpr1p, Pad1p and N-terminal), have been found in all but one the subunits of the lid; neither motif is present in the subunits of the base (Hofmann and Bucher 1998). The PCI domain is about 200 amino acids long with a predicted α -helix linked to a short loop structure and it is found in 5 subunits of the lid (Rpn3, Rpn5, Rpn6, Rpn7, Rpn9),

whereas the MPN domain is about 140 amino acids long with a predicted α/β structure and is found at the N-termini of the subunits Rpn8 and Rpn11. Rpn12 is the only subunit of the lid that contains neither of these two domains (reviewed by Voges et al. 1999).

The proteasome-dependent degradation pathway is ATP-dependent. It also usually involves the covalent attachment of ubiquitin, which is a highly conserved and ubiquitous eukaryotic 76-residue polypeptide that marks proteins targeted for degradation. Through the sequential activity of three enzymes, the activating E1, the conjugating E2 and the ligase E3 enzymes, an isopeptide bond is formed between the α -aminogroup of a lysine residue in the substrate protein and the carboxyl-terminus of the ubiquitin molecule. Subsequently, the ubiquitin itself is often ubiquitinated. This reaction is repeated with the subsequent formation of large polyubiquitinated substrates that are specifically recognised by the 26S proteasome complex (Figure 3.3) (reviewed by Ciechanover 1994; Hochstrasser 1995; Voges et al. 1999). Recently it was also reported that tetraubiquitin is the minimum signal for efficient targeting of substrate proteins for 26S proteasome-dependent degradation (Thrower et al. 2000).

The eukaryotic 26 S proteasome complex has three major peptidase activities:

- a chymotrypsine-like activity which cleaves after hydrophobic residues
- a trypsin-like activity which cleaves after basic residues
- a peptidyl-glutamyl peptide-hydrolysing activity which cleaves after acidic residues

Two additional activities, which cleave after branched chain residues and between small neutral amino acids have been identified in the mammalian proteasome (Voges et al. 1999).

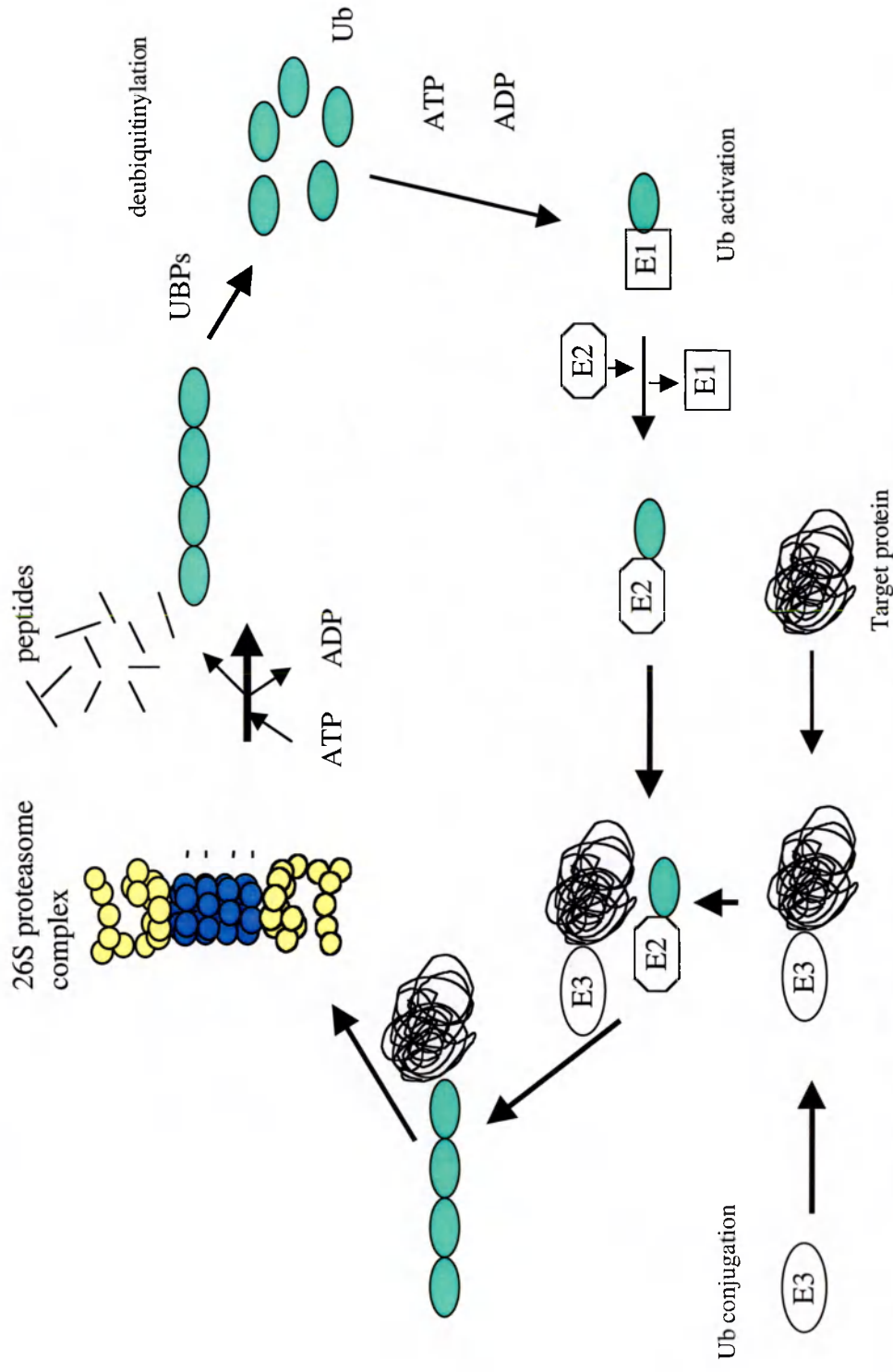


Figure 3.3 - Schematic representation of the ubiquitin-dependent degradation pathway

The multiprotein complex 26S proteasome is a ubiquitin-dependent degradation machinery. Ubiquitylation is a multistep dynamic process, during which a covalent attachment of ubiquitin to a substrate occurs. The activating enzyme E1 activates ubiquitin, which is then transferred to an ubiquitin conjugating enzyme E2. E2, in turn, transfers the ubiquitin molecule to the substrate by itself or in co-operation with the ubiquitin ligase enzyme E3. E3 binds directly to the substrate and confers specificity and regulation to the ubiquitylation process. In this way a polyubiquitin chain which targets the substrate for degradation is formed. After degradation by the 26S proteasome complex, ubiquitin molecules are recycled by UBPs enzymes in the so called deubiquitylation pathway.

The subcellular localization of the 26S proteasome

The 26S proteasome complex as well as the free forms 20S and 19S are localised in both the cytoplasm and the nucleus in mammalian cells and they appear to be excluded from the nucleoli (Peters et al. 1994). The nuclear localization of the 26S proteasome together with the high amount of the activating enzyme E1 and the presence of ubiquitin in the nucleus suggest important function for this pathway in the nucleus. Furthermore, it was also shown that the 26S proteasome is able to enter intact nuclei through the nuclear pores or during the re-assembly of the nuclear envelope after mitosis (Reits et al. 1997).

The 26S proteasome lid, COP9/signalosome and eIF3 complexes

PCI and MPN domains have been found exclusively in three multi-protein complexes, the 26S proteasome lid, the COP9 signalosome and the eukaryotic translation initiation factor 3 (eIF3) (Aravind and Ponting 1998; Hofmann and Bucher 1998) (Figure 3.4). Similarly to the lid of the 19S regulatory particle, all the eight subunits of the COP9 signalosome and 5 out eleven of the eIF3 complex contain the PCI and the MPN domains (Glickman et al. 1998a). Unfortunately, the biochemical functions of these domains remain unclear.

eIF3 is involved in the formation of the 43S translation preinitiation complex. It mediates the dissociation of the 80S ribosome into 40S and the 60S subunits and the interaction between the eIF2-GTP-Met-tRNA complex, the 40S ribosome and the messenger RNA (mRNA) (reviewed by Wei and Deng 1999).

The COP9 signalosome complex was first described in the plant *Arabidopsis* as a multisubunit repressor of photomorphogenesis and it is highly conserved among the eukaryotes (Wei et al. 1998). The mammalian COP9 signalosome was also identified

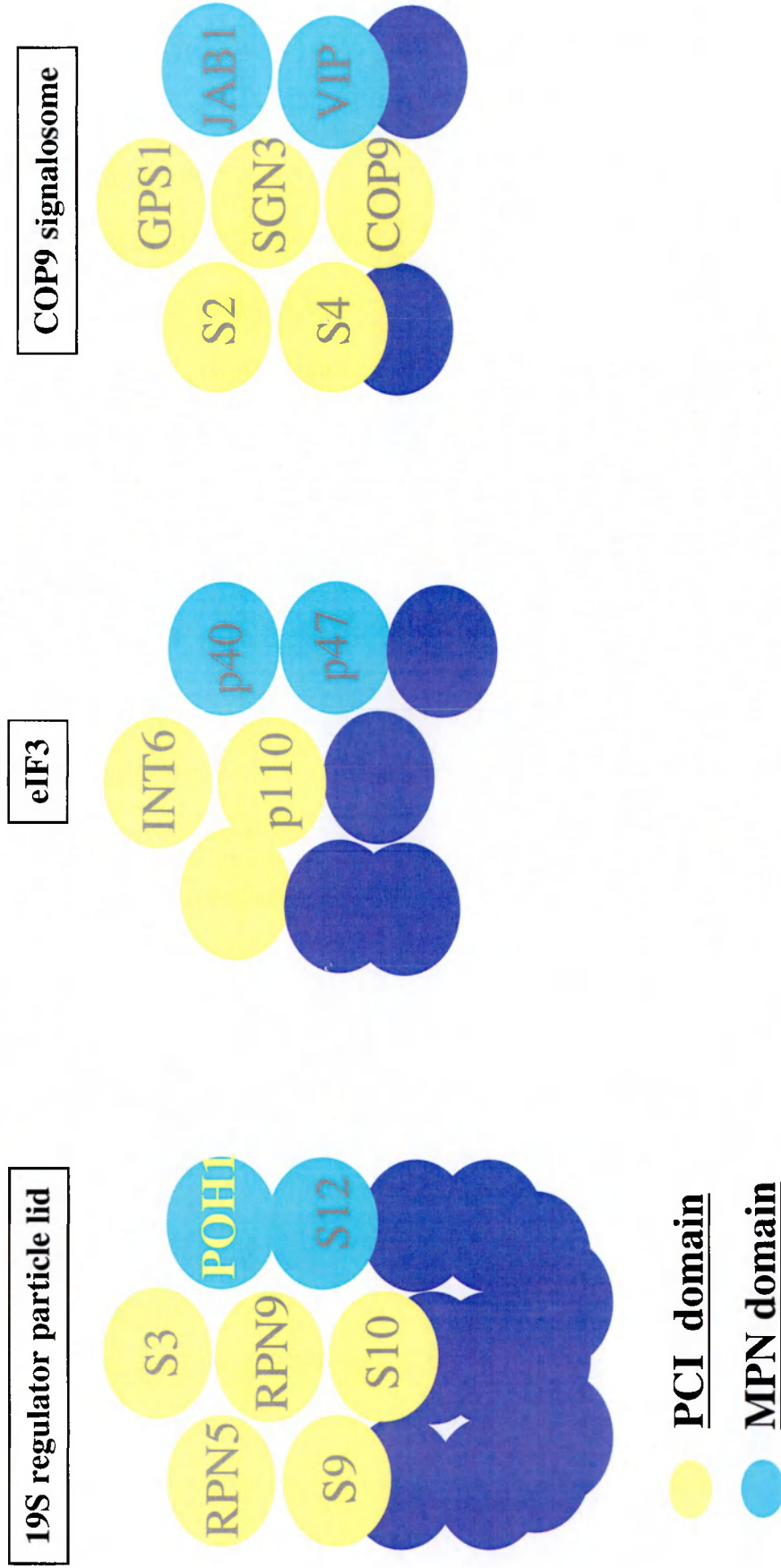


Figure 3.4 – Three multiprotein complexes containing PCI and MPN domains
 Similarity among the subunits of the lid of the 19S regulatory complex, the initiation translation factor eIF3 and the COP9 signalosome. The PCI (proteasome, COP9 and eIF3) and the MPN (Mpr1, Pad1 and N-terminal) domains are found only in the subunits of these multiprotein complexes.

by Seeger (Seeger et al. 1998) as a contaminating component of a 26S proteasome preparation from human erythrocytes. The complex was then separated from the 26S proteasome preparation and later it was purified from the pig spleen (Wei and Deng 1999).

It seems that the COP9 signalosome is involved in the regulation of several regulatory processes in the cell, such as transcription, signal transduction and protein degradation.

There is a one to one relationship between the subunits of the proteasome lid and the COP9 complex, suggesting that both complexes might derive from a common ancestral complex. Two models have been proposed (Figure 3.5) (Wei and Deng 1999):

- The 26S proteasome complex is the more ancient complex and the COP9 signalosome has evolved from that with the subsequent diversification of its function (Figure 3.5 model 1).
- An ancestral protein complex gave rise to both the COP9 signalosome and the 26S proteasome lid complexes (Figure 3.5 model 2).

Seeger *et al.* proposed that the homology among the subunits of these three multi-protein complexes may reflect a common substrate-binding site (Seeger et al. 1998); whereas Hofmann *et al.* suggested that the presence of the MPN and the PCI domains in the proteasome lid, eIF3 and COP9 signalosome complexes indicates that these complexes may act as a regulatory particle of the proteasome core complex (Hofmann and Bucher 1998). In support of this proposal, the COP9 signalosome complex was initially co-purified with the 26S proteasome complex (Seeger et al. 1998). Recently two of the eIF3 complex subunits, eIF3c (p48, also known as INT-6) and eIF3c

(p105), have been shown to interact with the COP9 signalosome complex in *Arabidopsis*. eIF3e subunit co-immunoprecipitates with the COP9 subunit CSN7 and the eIF3c subunit co-immunoprecipitates with the subunits eIF3b and eIF3e in the eIF3 complex and the subunits CSN1 and CSN8 in the COP9 complex (Yahalom et al. 2000). In addition, a direct interaction between eIF3e with eIF3c and CSN7 was also observed. This body of evidence suggests that although eIF3e and eIF3c are not components of the COP9 signalosome core complex, they are able to associate with the subunits of the COP9 signalosome. These interactions suggest a possible role for the COP9 signalosome in the control of protein translation (Yahalom et al. 2000).

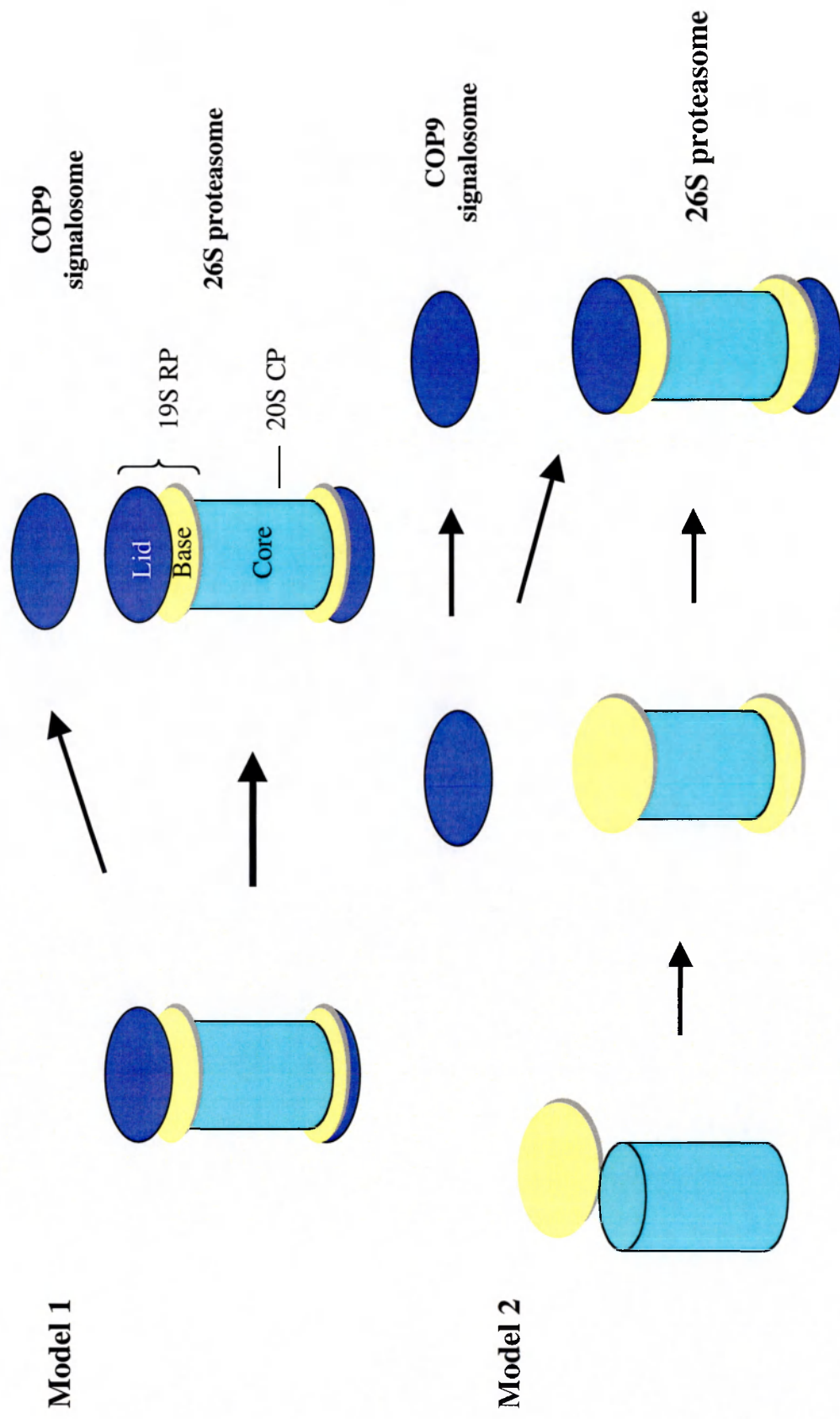


Figure 3.5 – Evolutionary origin of the 26S proteasome lid and the COP9 signalosome

Model 1. The 26S proteasome lid is the ancestral complex from which the COP9 signalosome evolved and assumed a new function in the cell.

Model 2. A protein complex ancestor gave rise to both the 26S proteasome lid and the COP9 signalosome.

Results

Constitutive stable expression of *HA-POH1* in monkey COS7 cells

In order to investigate the multi-drug resistance phenotype conferred by overexpression of the proteasomal regulatory subunit Pdh1, stable over-expression of *HA-POH1* in monkey COS-7 cells was initially pursued.

POH1 fused to the 3'-end of the single HA epitope-tag was previously cloned into the pcDNA3 mammalian expression vector (pcDNA3/HA-POH1) (Spataro et al. 1997), which drives the expression of the gene under the control of the cytomegalovirus (CMV) immediate early promoter. The vector also contains a SV40 virus replication origin, which allows episomal replication of the vector in the presence of the SV40 large T antigen and a higher expression level of the cloned gene.

COS-7 cells were co-transfected with the pOPRSV/CD2 plasmid, which expresses a truncated version of the rat CD2 protein as a cell surface marker of transfection, and the plasmid pcDNA3/HA-POH1 or the empty vector pcDNA3.1+, at a ratio of 1:4. It was assumed that most of the cells expressing CD2 after transfection would also contain the empty vector or pcDNA3/HA-POH1 respectively. After transfection the CD2⁺ cells were selected using the immunomagnetic beads coated with a mouse monoclonal anti-CD2 antibody (Figure 3.6). The CD2⁺-selected cells were then selected for the stable maintenance of the co-transfected vector (pcDNA3.1+ or pcDNA3/HA-POH1) for two weeks with the antibiotic Geneticin (G-418). Expression of the HA-Pdh1 protein was then tested. Whole cell lysates were prepared from the G-418-resistant polyclonal cells transfected with the empty vector or with pcDNA3/HA-POH1. The detection of HA-Pdh1 was carried out by immunoblot analysis using an HRP-conjugated monoclonal anti-HA antibody (Figure 3.7A). A band of about 37 kDa

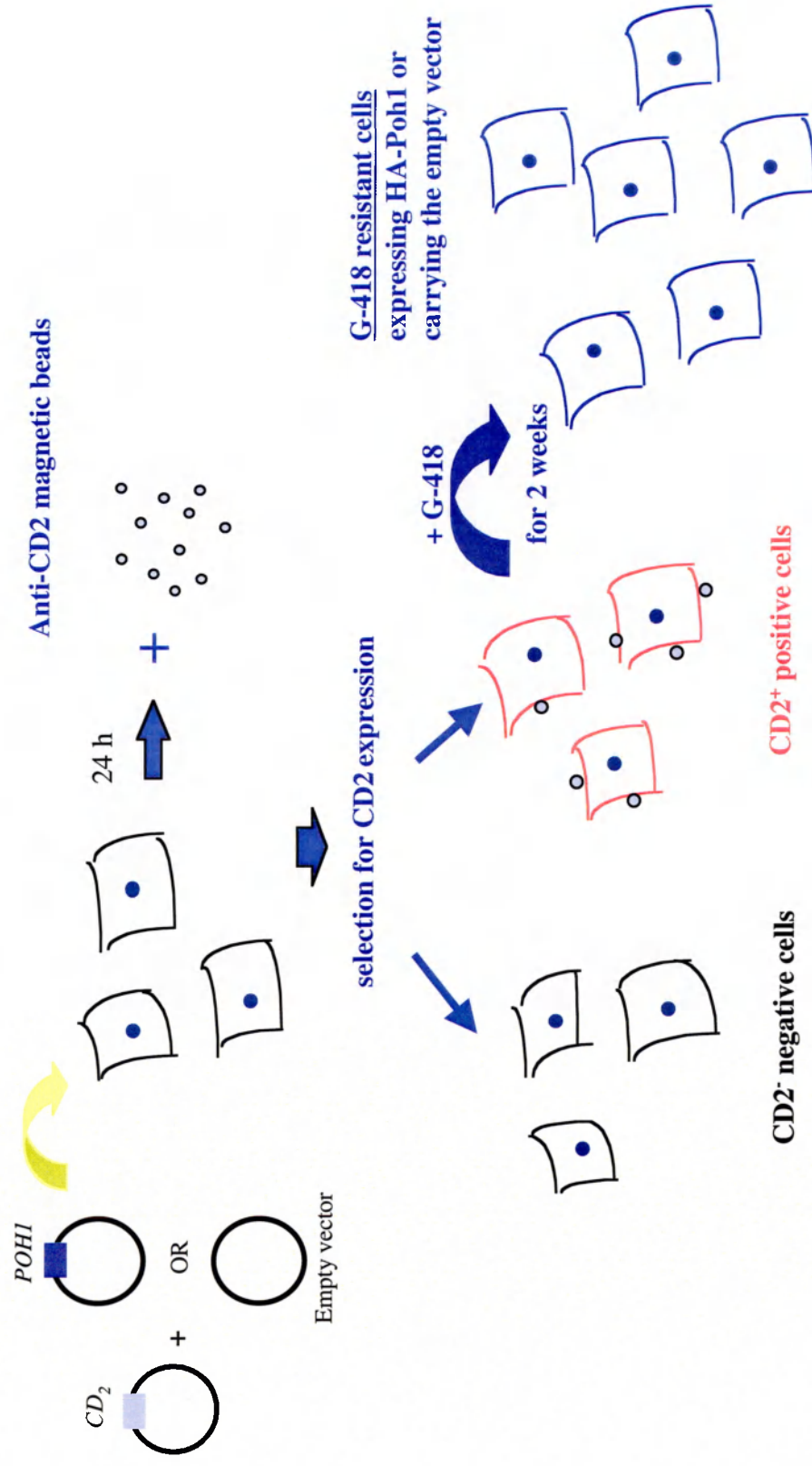


Figure 3.6 – Selection of CD2-expressing cells

COS7 cells were co-transfected with the empty vector or pcDNA3/HA-POH1 and pOPRSV/CD2. 24 hours later the cells were selected for the expression of the CD2 surface as a transfection marker (CD2⁺) by immunomagnetic beads coated with mouse monoclonal anti-CD2 antibody. The CD2⁺-selected cells were then plated and selected for the resistance to the pcDNA3-selective antibiotic G-418 for two weeks.

was detected in the cell lysates from the HA-Poh1-transfected cells. The Cdc2 protein was also detected as loading control using a mouse monoclonal anti-Cdc2 (A17) antibody. Figure 3.7B shows the expression of both HA-Poh1 and Cdc2 proteins in two positive stable clones, 2 and 25, which were derived from the stable polyclonal pool (see Materials and Methods - Chapter 2). Clone 2 cells showed the highest HA-Poh1 expression level when compared with cells from clone 25 or from polyclonal pool.

HA-Poh1 stable expression leads to drug resistance

A comparison between HA-Poh1 endogenous expression levels and the cellular responses to 175 compounds in clinical use was carried out using the NCI 60 cancer cell line panel. A significant positive correlation was observed between high endogenous Poh1 protein level in these cell lines and resistance to 36 of the cytotoxic drugs (C.Norbury, personal communication). Platinum drugs, alkylating agents and topoisomerase II inhibitors were among these drugs with the strongest correlation (Table I).

With these correlative data in mind, I investigated if the overexpression of HA-Poh1 in the COS-7 cells was able to lead to resistance to a wider range of drugs than those previously tested (7-hydroxystaurosporine and taxol) (Spataro et al. 1997). To this end cisplatin, doxorubicin and melphalan were chosen as representative of the classes of drugs listed in Table I. The susceptibility of the cells to these drugs was tested by comparing the clonogenic survival in COS-7 cells stably expressing HA-Poh1 or carrying the empty vector. Cells from the pcDNA3.1+ or the pcDNA3/HA-POH1 polyclonal pools were exposed to different concentrations of cisplatin, doxorubicin or melphalan for 24 hours and the capacity to form colonies was measured after 12-14

days. Figure 3.8 shows the results of two independent experiments carried out in triplicate where the HA-Poh1-overexpressing cells have a survival advantage after exposure to cisplatin (A) and melphalan (B) in comparison with the cells carrying the empty vector. The HA-Poh1 overexpressing polyclonal cells do not show any significant resistance to doxorubicin (C).

Table I - Analysis of variance shows positive correlation between endogenous Poh1 level and resistance to cytotoxic drugs in the NCIH 60 cell line panel. Compounds listed in order of correlation coefficient.

Chemical name	Pearson Corr Coef	P (Two-tail)
CBDCA (Carboplatin)	-0.463	1.9E-04
Cis-platinum	-0.426	1.8E-04
Deoxydoxorubicin	-0.408	1.2E-03
Largomycin	-0.396	1.7E-03
Pipobroman	-0.395	1.8E-03
Triethylenemelamine	-0.387	2.2E-03
Thio-tepa	-0.384	2.4E-03
Mytomycin C	-0.374	3.3E-03

In addition, the drug sensitivity of the cells from the two stable clones, 2 and 25, was examined. The MTS cytotoxicity assay was chosen because the cells from clone 2 showed slow growth rate and low plating efficiency; however, no difference in the cell cycle phase distribution was observed (data not shown). The attempts to test the

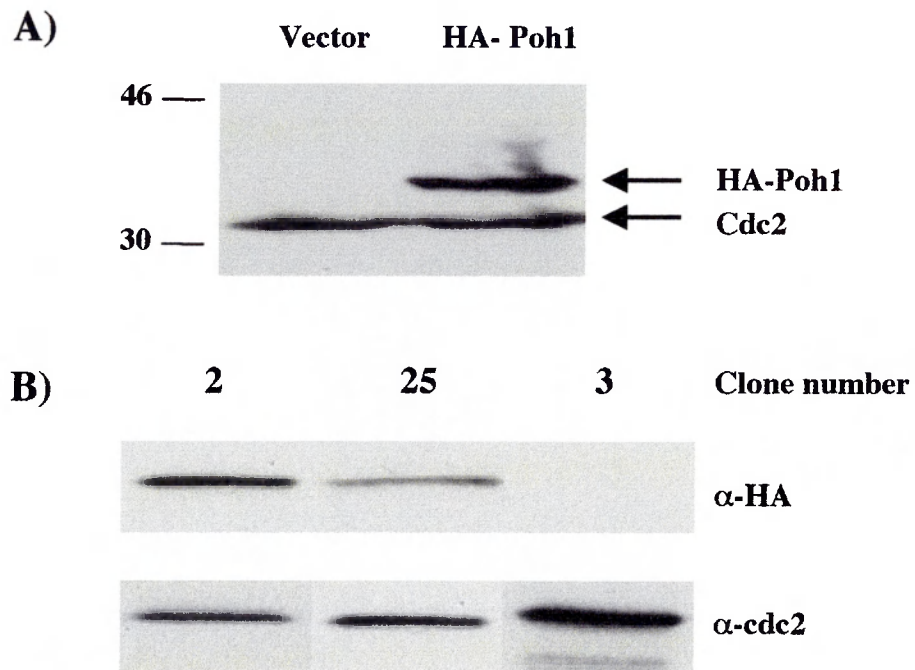


Figure 3.7 – HA-Poh1 stable overexpression in COS7 polyclonal pool and in two clones

Immunoblot of the exogenous expression of HA-Poh1 in COS7 cells from polyclonal pool (A) or clones (B) stably transfected with pcDNA3/HA-POH1 or the empty vector. Overexpressed protein was detected from whole cells extracts using HRP-conjugated monoclonal anti-HA antibody. For loading control mouse anti-Cdc2 monoclonal antibody (A17) was used.

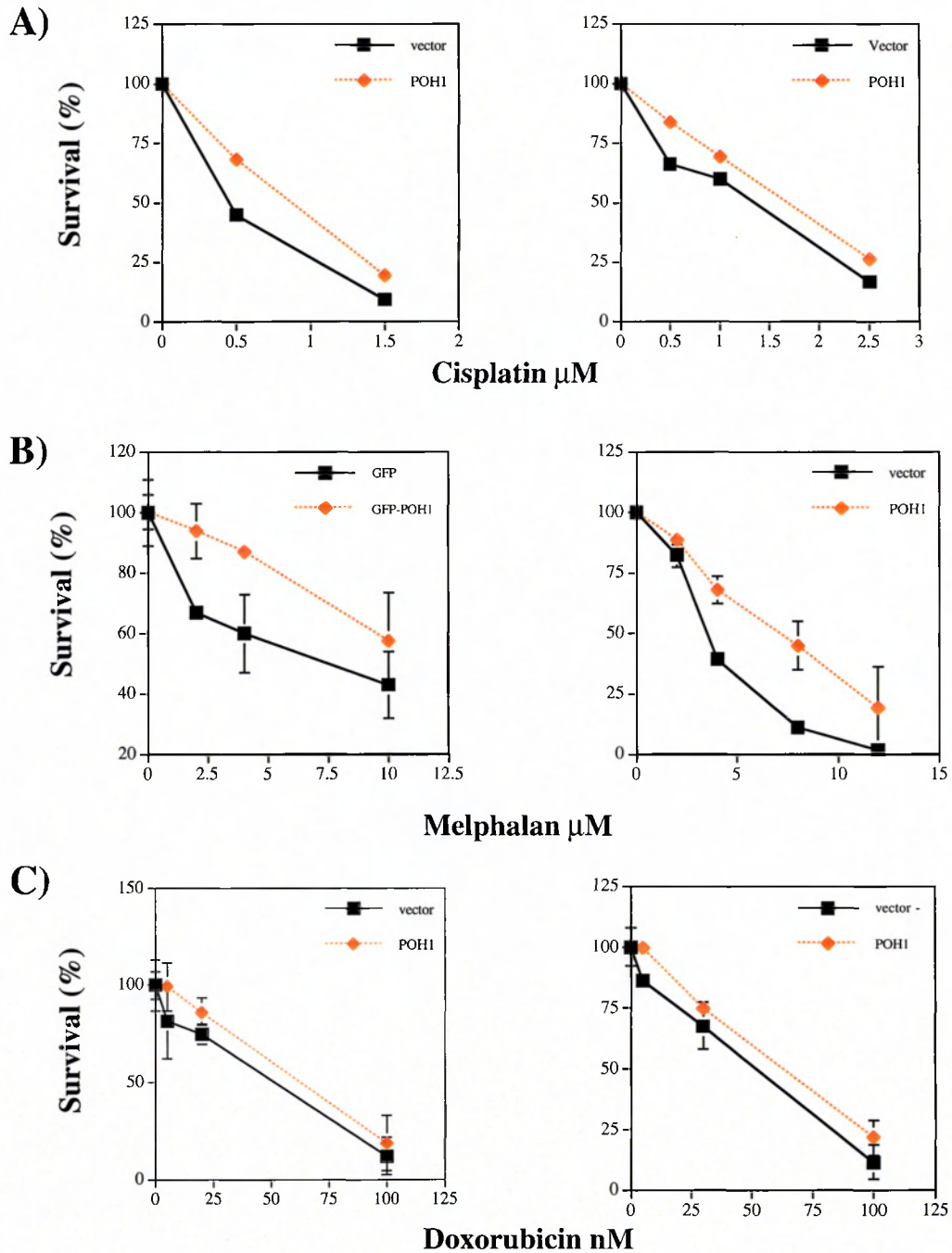


Figure 3.8 - HA-Poh1 stable overexpression confers resistance to cisplatin and melphalan

Clonogenic survival curve in COS7 stably transfected with the empty vector (pcDNA3.1+) or the pcDNA3/HA-POH1 plasmid. Cells were plated in triplicate and treated with different concentrations of cisplatin (A) or melphalan (B) or doxorubicin (C) for 24 hours. The cells were washed once with PBS and then refed with fresh medium.

The number of surviving colonies was counted after two weeks and expressed as the percentage of survival compared to the control (cells with no drug). SD is not shown when smaller than the plot symbol. Two experiments for each drug treatment are shown.

responses of these cells to drug treatment using the clonogenic assay were unsuccessful. The cells from clone 2 and clone 25 and polyclonal pool carrying the empty vector were treated with several dilutions of cisplatin or doxorubicin for 72 hours (Figure 3.9). A much higher range of drug concentrations was chosen in the MTS assay as the same range used in the clonogenic assay was ineffective. Resistance to both drugs was also observed in the two HA-Poh1-overexpressing clones. The higher resistance observed in figure 3.9 compared to the figure 3.8 is possibly due to the different approaches used. The MTS assay measures the metabolically active cells whereas the clonogenic assay measures the capacity of the cells to proliferate and to form colonies. Furthermore, the cells in MTS assay were analysed after a much shorter time than in the clonogenic assay.

Association of Poh1 with the 26 S proteasome complex

Given that HA-Poh1 was described as a regulatory subunit of the 26S proteasome complex (Spataro et al. 1997), the possible association of the exogenous HA-Poh1 with the 26S proteasome complex was investigated. To this end, protein complexes were separated by 7-37% sucrose density gradient centrifugation of extracts from polyclonal COS-7 cells stably expressing HA-Poh1 (by R.Murray). Aliquots from the gradient fractions were subsequently used for immunoblot analysis (Figure 3.10). Exogenous and endogenous Poh1 were detected using HRP-conjugated monoclonal anti-HA and rabbit polyclonal anti-HA-Poh1 CN13 antibodies, respectively (Spataro et al. 1997). The endogenous protein was found in the fractions 6 and 7 where the 19S regulatory subunit p42 was also detected, suggesting that these fractions contained the 26S proteasome complex. Surprisingly, most of the overexpressed HA-Poh1 was

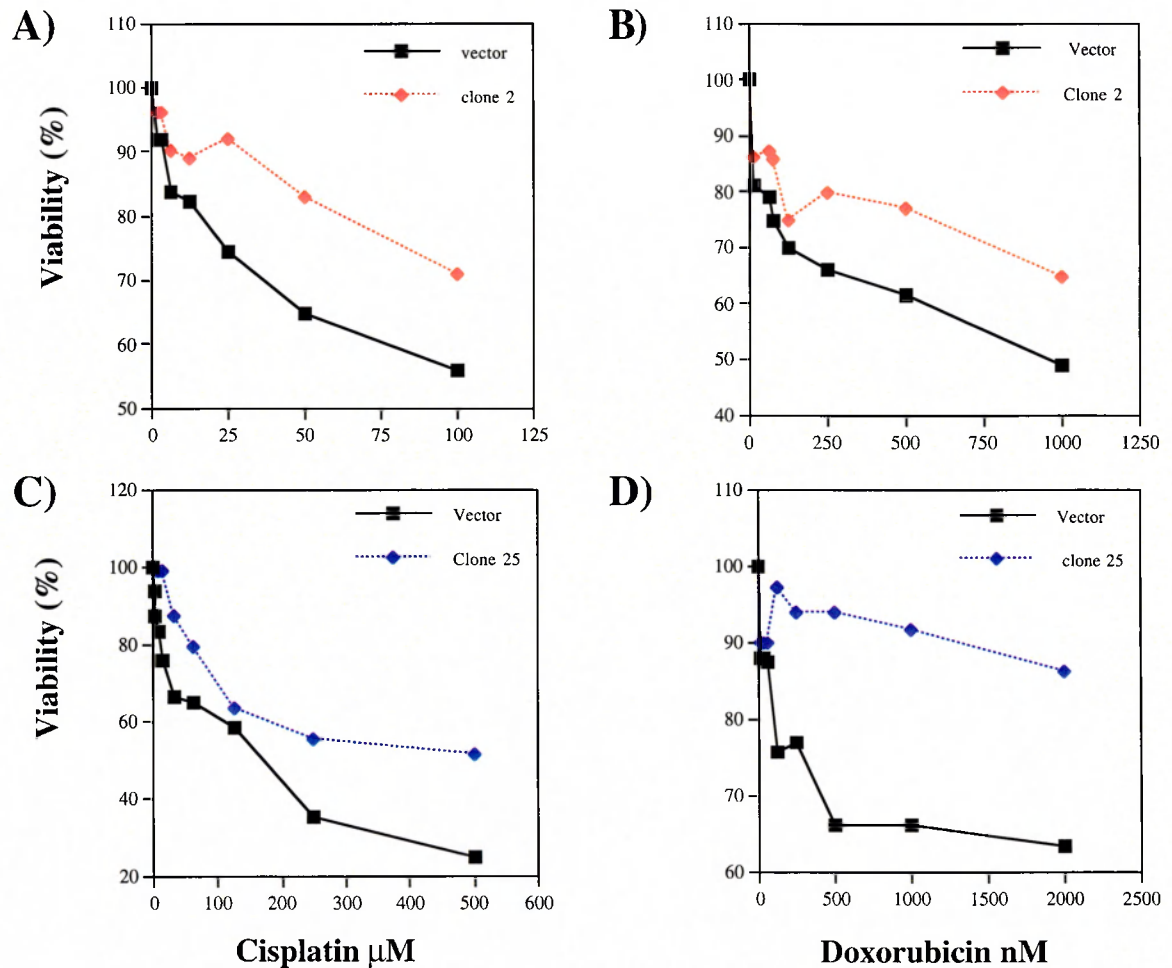


Figure 3.9 – HA-Poh1 overexpression in clones 2 and 25 confers resistance to cisplatin and doxorubicin

MTS cytotoxicity assay in HA-Poh1 expressing stable clones. 1000 cells were plated in triplicate in 96-multiwell plates. 24 hours later the cells from clone 2 (A,B) and clone 25 (C,D) were treated with serial dilutions of cisplatin (A,C) or doxorubicin (B,D) for 72 hours. Cell viability was scored by measuring the absorbance at 490 nm using a plate reader. Results are expressed as percentage of viability compared to the control (no drug).

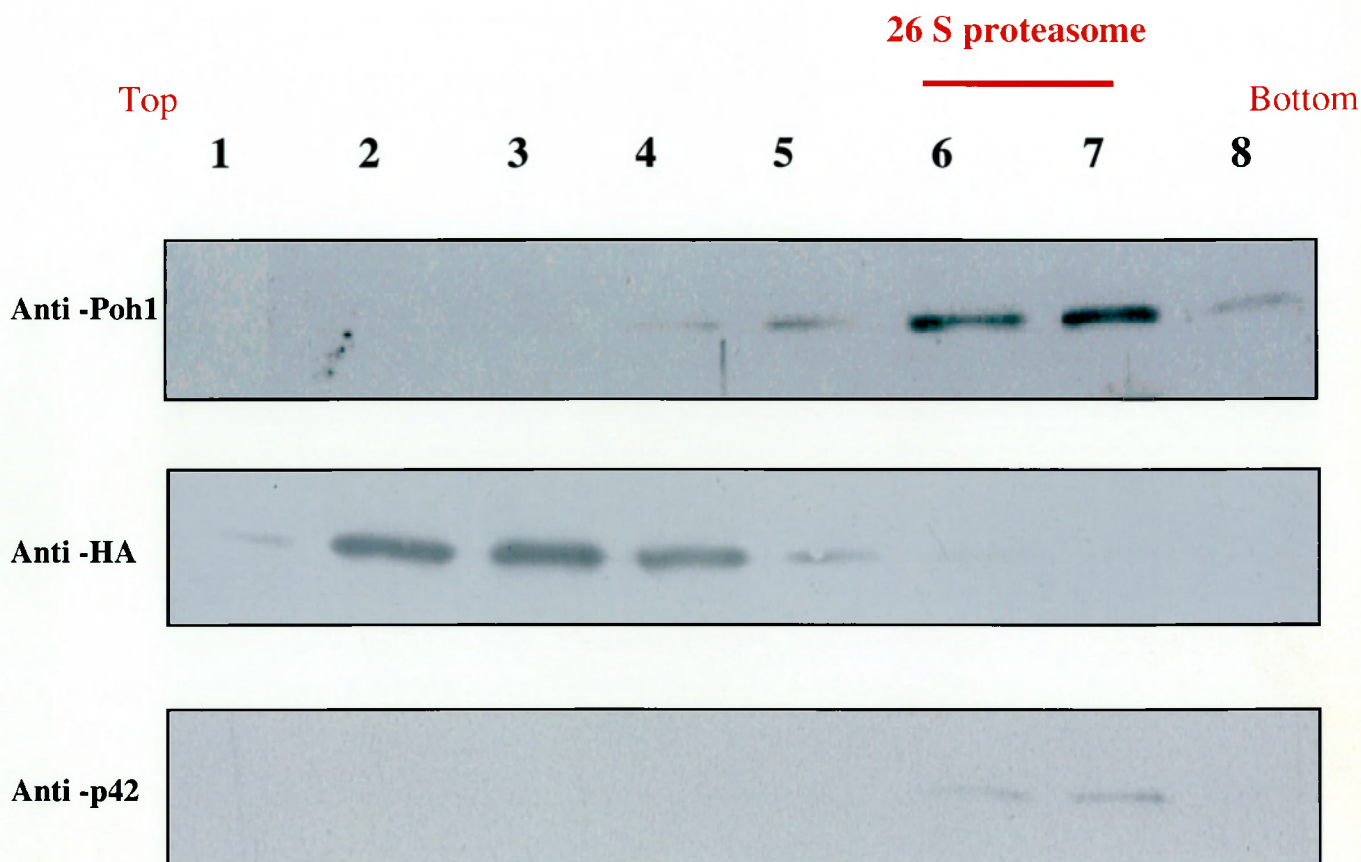


Figure 3.10 – Overexpressed HA-Poh1 is incorporated into low molecular weight complexes

7-37% sucrose density gradient centrifugation of cell lysates from COS-7 polyclonal cells stably expressing HA-Poh1. Detection of the exogenous and endogenous Poh1 was carried out by immunoblot analysis from the gradient fractions 1 to 8 using HRP-conjugated monoclonal anti-HA and polyclonal anti-Poh1 CN13 antibodies, respectively. The proteasome regulatory subunit p42 was detected as a positive control using monoclonal anti-p42 antibody.

detected in the lower molecular weight fractions (2 to 5) with almost undetectable amount in fraction 6.

In order to further investigate the association between the exogenous HA-Poh1 and the proteasomal complex, the core (20S) subunit C3 of the 26S proteasome was immunoprecipitated from extracts of COS-7 cells carrying the empty vector or overexpressing HA-Poh1 using the mouse monoclonal MCP-21 (multicatalytic protease) antibody (Hendil et al. 1995). HA-Poh1 and C3 were then detected by immunoblot analysis. Lysis buffer alone was used as negative control for the immunoprecipitation. A faint band corresponding to exogenous HA-Poh1 was detected only in HA-Poh1-containing cell lysate (Figure 3.11A), whereas C3 protein was detected in both cell lysates (Figure 3.11B). The results described so far point towards a poor association of the exogenous HA-Poh1 with the 26S proteasome complex.

Next, the amount of HA-Poh1 associated or non-associated to the proteasomal complex was detected. To this end, immunoprecipitation of the core subunit C3 was performed again with the difference that in addition to the C3-precipitated sample the supernatant (un-precipitated) material was also collected after immunoprecipitation. The samples were then subjected to immunoblot analysis. Figure 3.12A shows a band corresponding to the overexpressed protein that is detectable in both the precipitated and the supernatant samples only from the HA-Poh1-overexpressing cells. Considering the amount from each sample loaded on the gel in respect to the total, the results shown in figure 3.12 indicate that most of the overexpressed HA-Poh1 does not associate with the 26S proteasome complex.

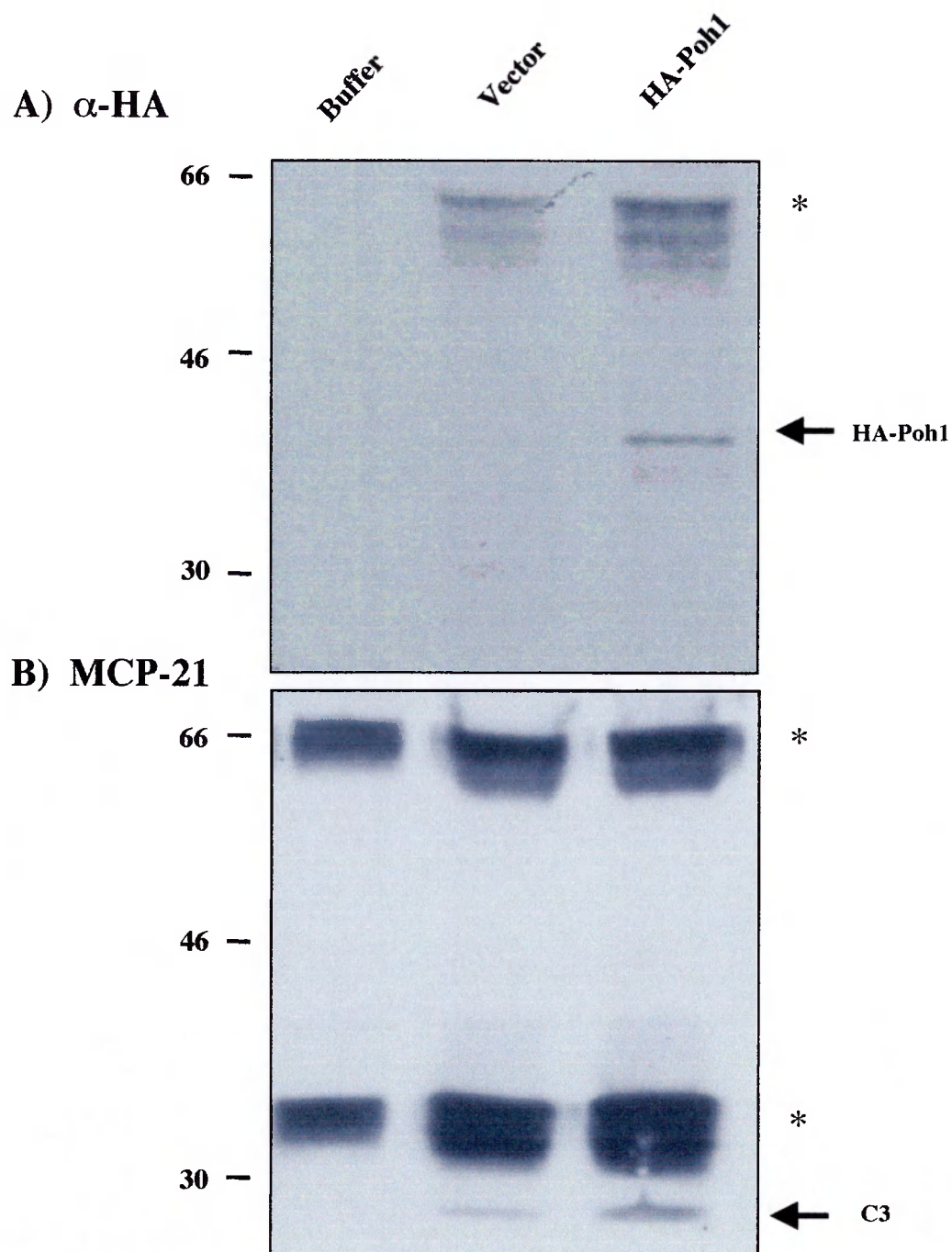


Figure 3.11 - Exogenous HA-Poh1 co-precipitates with the proteasome core subunit C3

Immunoprecipitation of the 20S proteasome core subunit C3 from the COS7 cells carrying the empty vector or overexpressing HA-Poh1 was performed using the mouse monoclonal MCP-21 antibody. Detection of HA-Poh1 (A) and C3 (B) proteins was carried out using mouse monoclonal MCP-21 and HRP-conjugated monoclonal anti-HA antibodies, respectively. Lysis buffer alone was used as negative control.

* IgG

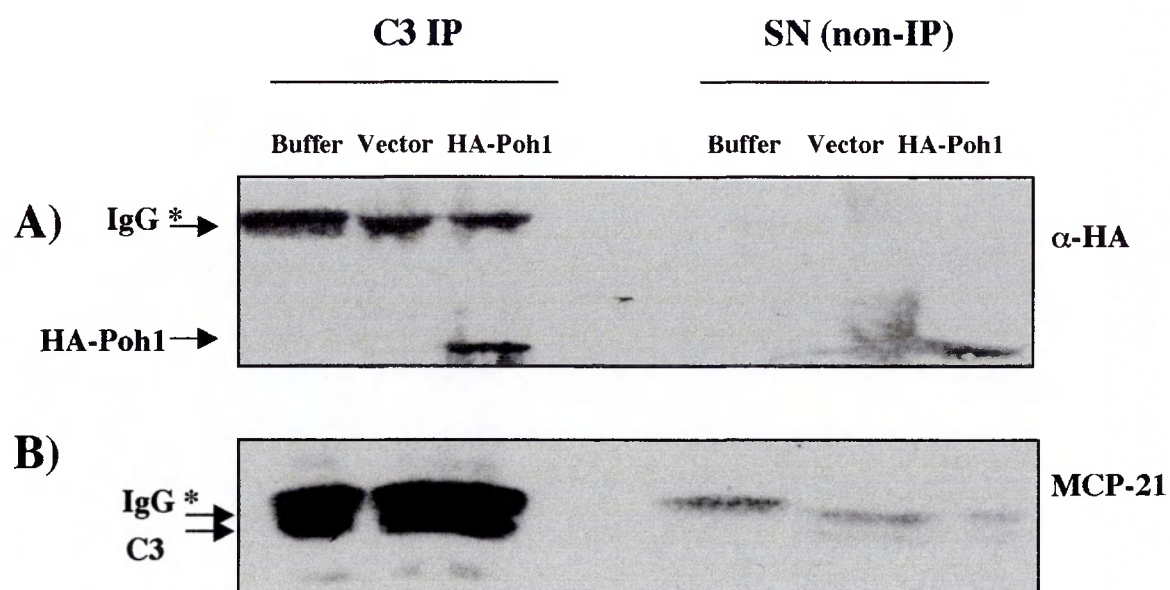


Figure 3.12 – Most of the exogenous HA-Poh1 is not proteasome-associated

Immunoprecipitation of 20S proteasome core subunit C3 from COS7 cells stably transfected with pcDNA3.1+/HA-POH1 or the empty vector was performed using mouse monoclonal MCP-21 antibody. Poh1 (A) and C3 (B) proteins were detected in the precipitated and unprecipitated samples using HRP-conjugated monoclonal anti-HA and mouse monoclonal MCP-21 antibodies, respectively. Lysis buffer alone was used as negative control.

Sub-cellular localization of HA-Poh1 in COS-7 cells

The localization of the exogenous HA-Poh1 in COS-7 cells was studied by using both direct fluorescence and indirect immunofluorescence approaches.

First, expression of *POH1* was carried out using the pEGFP expression vector. *POH1* cDNA was fused to the 3'-end of the enhanced green fluorescence protein (EGFP) DNA sequence in the pEGFP vector. pEGFP empty vector and pEGFP/POH1 were then transiently expressed in COS-7 cells were carried out. Expression of the fusion protein was confirmed in whole cell lysates by immunoblot analysis using a rabbit polyclonal anti-EGFP antibody (Figure 3.13). A band corresponding to the EGFP-tagged HA-Poh1 protein (lane 2) was identified. In line with the expected molecular mass of 37 kDa for Poh1 and of about 30 kDa for EGFP (lane 1), the EGFP-Poh1 fusion protein migrated at 60-62 kDa. Next, the localization of the expressed proteins was analysed. The cells were grown on sterile glass coverslips in a 6-multiwell plate and the pEGFP or the pEGFP-POH1 vectors were transiently expressed with an efficiency of approximately 80-90%. As detected by fluorescence microscopy, the EGFP-Poh1 protein localised mainly in the cytoplasm with low or undetectable localization within the nucleus (Figure 3.14, second and third columns). The same result was obtained after transient transfection of the pEGFP-POH1 plasmid in human HeLa cells (data not shown). EGFP alone is known to be able to passively diffuse through the nuclear membrane and localises throughout the cytoplasmic and nuclear compartments (Figure 3.15).

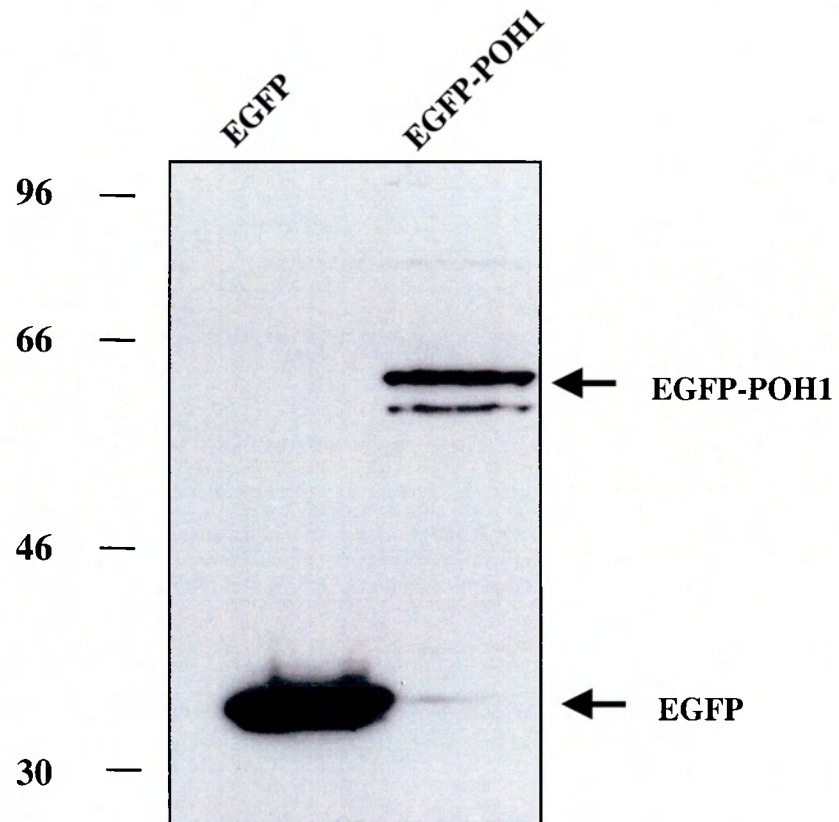


Figure 3.13 - EGFP and EGFP-Poh1 protein detection in COS-7 cells

COS-7 cells transiently transfected with pEGFP or pEGFP-POH1 were lysed in 2x SDS sample buffer and lysates were separated by 10% SDS-PAGE. EGFP and EGFP-Poh1 detection was carried out by immunoblot analysis using rabbit polyclonal anti-GFP antibody.

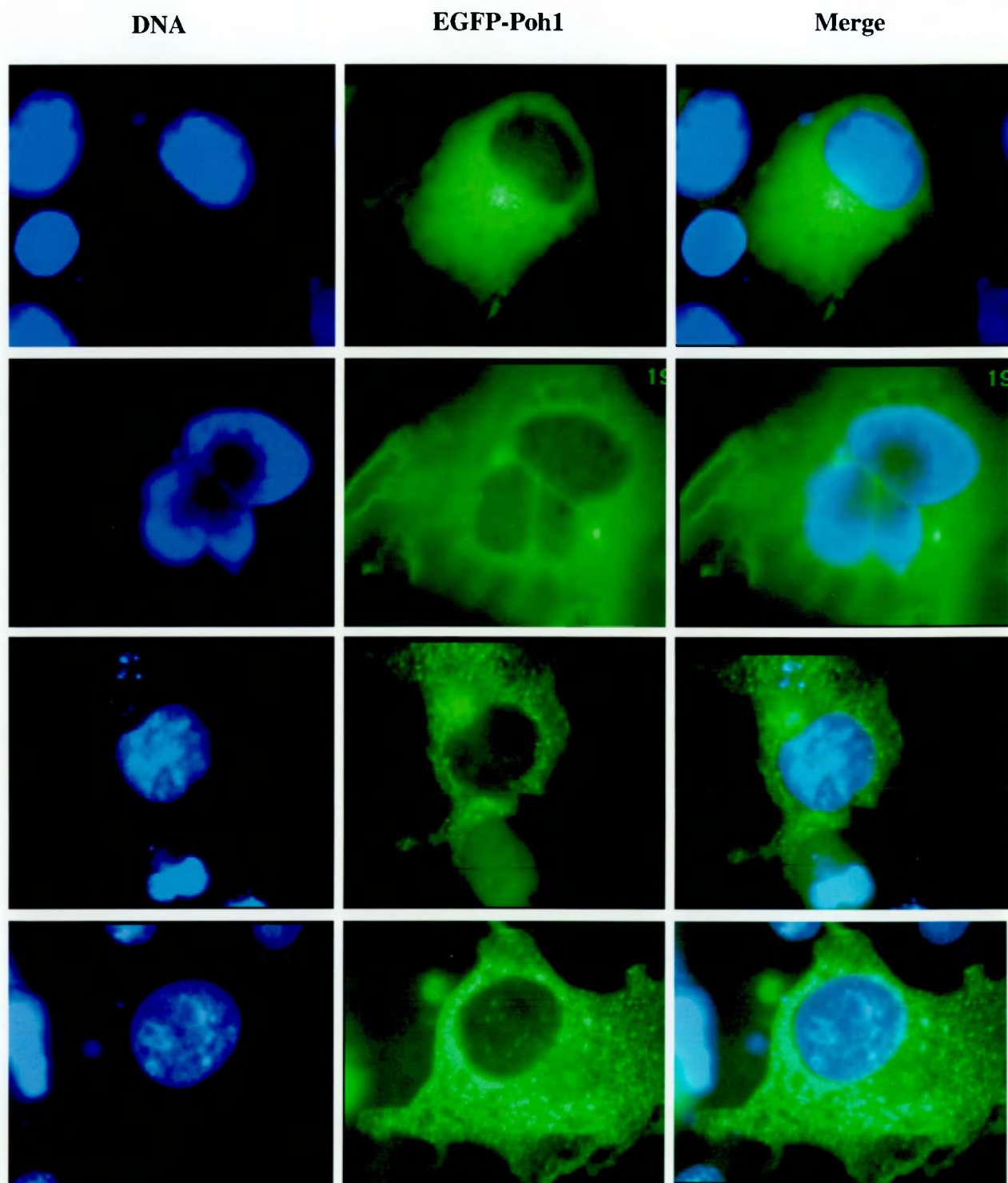


Figure 3.14 – Transiently expressed EGFP-Poh1 is localised in the cytoplasm of COS-7 cells

COS-7 cells were grown in sterile glass coverslips in a 6-multiwell plate. Cells were transiently transfected with pEGFP/POH1 and 24 hours later were fixed with 4% paraformaldehyde. DNA was stained using Hoechst 33258 [1µg/ml] and localization of the EGFP-tagged recombinant protein was detected by fluorescence microscopy.

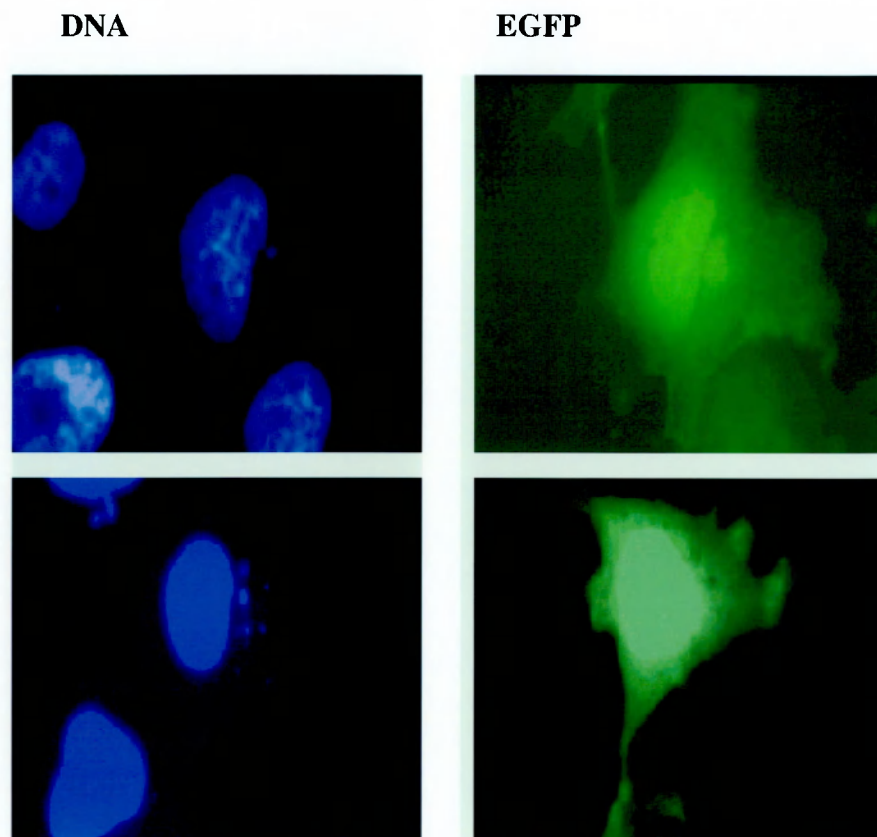


Figure 3.15 - EGFP localization in COS-7 cells

COS-7 cells were grown in sterile glass coverslips in a 6-multiwell plate. Cells were transiently transfected with the pEGFP vector and 24 hours later were fixed with 4% paraformaldehyde. DNA was stained using Hoechst 33258 [$1\mu\text{g/ml}$] and localization of EGFP was detected by fluorescence microscopy.

Surprisingly, when the stable expression of HA-Poh1 in COS-7 cells was investigated by indirect immunofluorescence, the result was different from that obtained with the EGFP fusion. The cells were grown on sterile glass coverslips in a 6-multiwell plate. 24 hours after plating, the localization of HA-Poh1 was determined using mouse monoclonal anti-HA and anti-mouse FITC-conjugated antibodies (Figure 3.16A); anti-mouse FITC-conjugated antibody was used alone as a negative control (Figure 3.16B). Co-localization of the HA-Poh1 fluorescence signal and the nucleus was observed in addition to the cytoplasmic localization of the protein.

These data were further supported by sub-cellular fractionation (Figure 3.17). Soluble (S) and nuclear enriched (N) fractions of COS-7 polyclonal pools carrying the empty vector or overexpressing HA-Poh1 were prepared as described for the nuclear extracts (Dignam et al. 1983) used for the electrophoretic mobility shift assay (see Materials and Methods - Chapter 2) with the difference that after cell lysis and separation from the nuclei the soluble fraction was also collected. The samples were then subjected to immunoblot analysis. Exogenous and endogenous HA-Poh1 was detected in the nucleus and the cytoplasm by using HRP-conjugated monoclonal anti-HA and polyclonal anti-HA-Poh1 CN13 antibodies, respectively. Overexpressed and endogenous Poh1 were both detected in the both fractions with the majority of the signal being soluble.

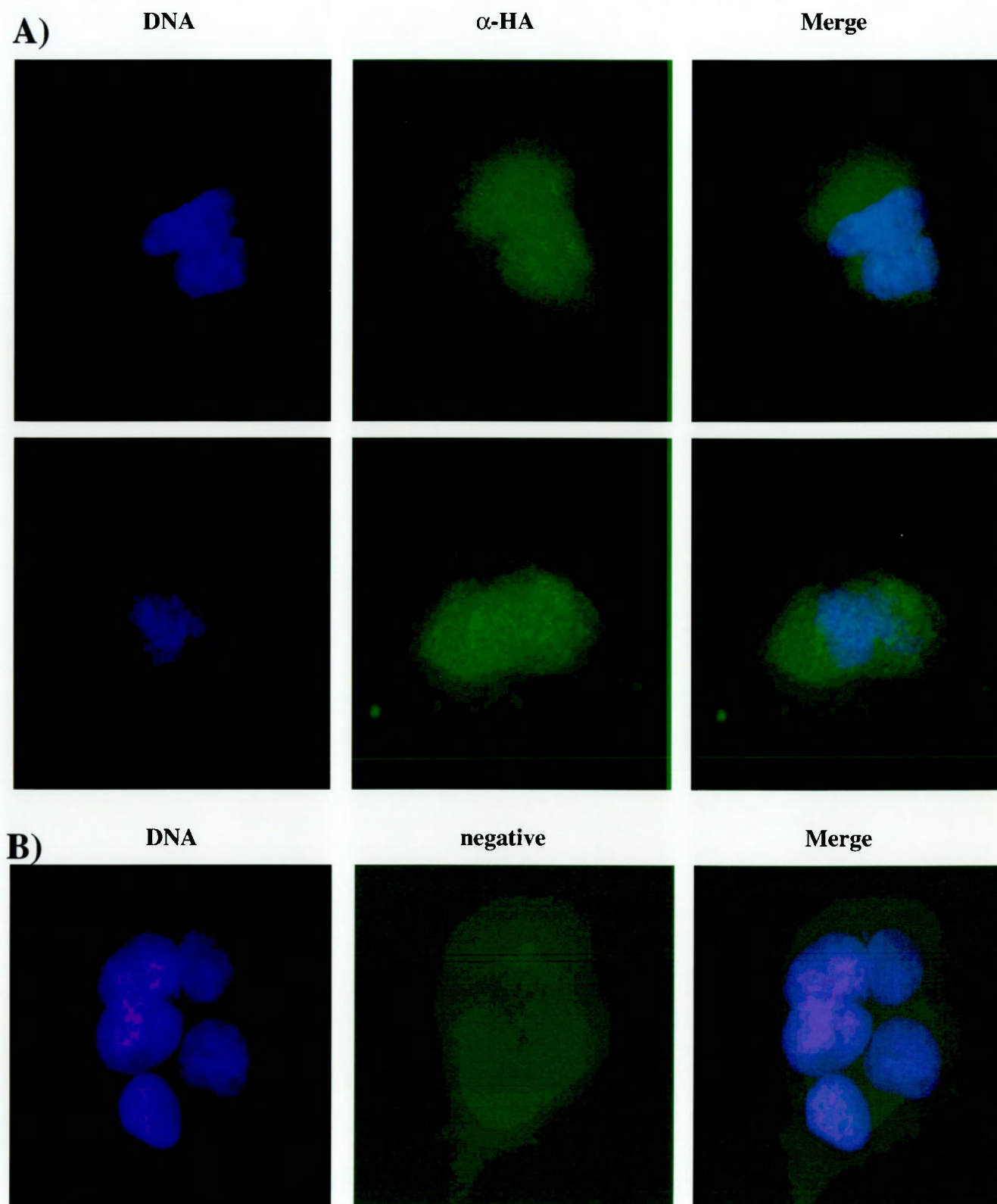


Figure 3.16 – Exogenous HA-Poh1 is localized in the nucleus and cytoplasm of COS-7 cells
 Indirect immunofluorescence of COS-7 cells from polyclonal population stably overexpressing HA-Poh1. Cells were grown on sterile glass coverslips in a 6 multiwell plate for 24 hours, before being fixed in 4% paraformaldehyde, permeabilised in 0.5% Triton X-100 and stained using mouse monoclonal anti-HA antibody and anti-mouse FITC-conjugated secondary antibodies (A) or anti-mouse FITC-conjugated antibody only (B), as a negative control. DNA was stained with Hoechst 33258 [1 μ g/ml], the cell were then mounted in 90% glycerol and fluorescence was detected by fluorescence microscopy.

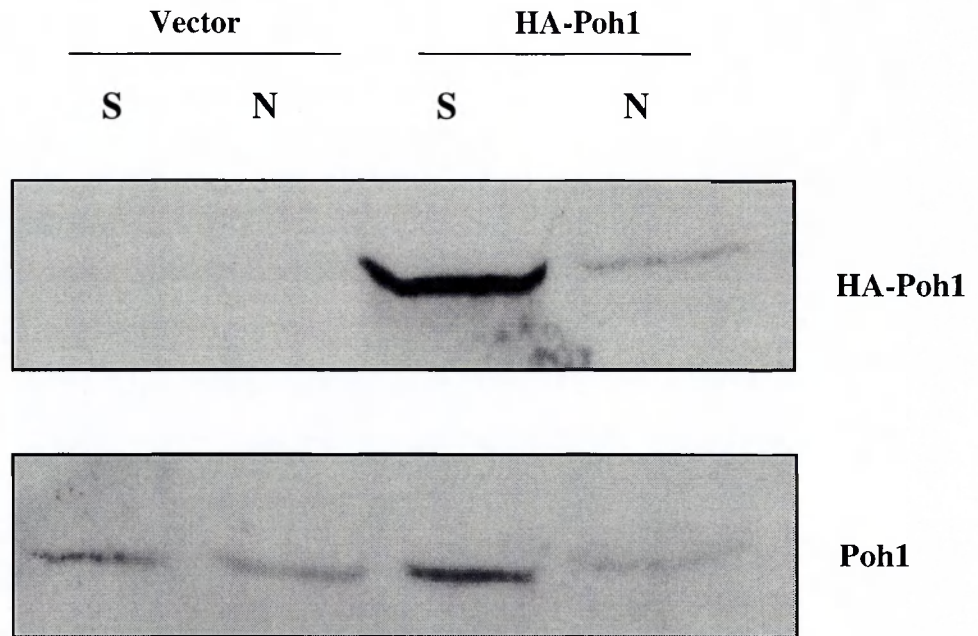


Figure 3.17 – Endogenous and exogenous Poh1 are expressed both in the soluble and the nuclear enriched fractions

Detection of HA-Poh1 by immunoblot analysis of nuclear and soluble fractions from COS-7 cells stably expressing HA-Poh1 or carrying the empty vector. A) Exogenous HA-Poh1 was detected using HRP-conjugated monoclonal anti-HA antibody, whereas B) endogenous Poh1 was detected using rabbit polyclonal anti-Poh1 CN13 antibody.

Discussion

HA-Poh1 constitutive expression and drug resistance

In order to elucidate the mechanism of drug resistance induced by HA-Poh1, stable expression of HA-Poh1 was obtained in a polyclonal pool population and in two clones, 2 and 25. Unfortunately, the expression level of the exogenous HA-Poh1 was not high when compared to the level of the endogenous protein, which was detected using the polyclonal anti-Poh1 CN13 antibody. On the other hand, this level of exogenous HA-Poh1 was able to confer resistance to different drugs in COS-7 cells. The survival advantage of the HA-Poh1-overexpressing cells following exposure to cisplatin and melphalan was observed in the polyclonal pool and to cisplatin and doxorubicin in the single cell clones. The drugs chosen for these experiments have different mechanisms of action. Cisplatin forms DNA adducts, either inter- or intra-molecular, causing DNA damage; doxorubicin is an anthracycline which inhibits topoisomerase II and binds directly to the DNA possibly by direct intercalation into the DNA strand with subsequent inhibition of the DNA synthesis; and melphalan belongs to the group of alkylating agents, which exert a wide range of biological effects possibly mediated by alkylation of the guanine in the DNA (reviewed by Brown and Boger-Brown. 1999).

It is worthy to note that the cells from the stable clone 2, which had the highest HA-Poh1 expression level, also exhibited morphological changes and growth defects. Cultures of this clone grow slowly and contain many cells that appear to be senescent. The cells also appear generally larger and flatter than the un-transfected cells or other HA-Poh1-transfected cells. It is unclear if these growth and morphological changes are

due to the high expression level of HA-Poh1, rather than HA-Poh1-independent clonal variation.

Sub-cellular localization of HA-Poh1

Stably expressed HA-Poh1 is found both in the cytoplasmic and in the nuclear compartments of COS-7 cells, as shown by indirect immunofluorescence (Figure 3.16B). No major difference of intensity between the two compartments was observed. This result is in accord with other studies on the localization of the 26S proteasome complex in mammalian cells. Both the 26S complex and its 20S and 19S constituents have been found in the nucleus and the cytoplasm of mammalian cells (Peters et al. 1994). However, it is still unclear if the process of proteolysis occurs in both compartments or specifically in one. There are no data regarding the localization of the endogenous Poh1, as the rabbit polyclonal anti-Poh1 CN13 antibody does not recognise the protein by immunofluorescence (Spataro et al. 1997). Endogenous HA-Poh1 is mainly associated with the 26S proteasome complex as detected in crude lysates of human cells (Spataro et al. 1997) and in the COS-7 cells overexpressing HA-Poh1 (Figure 3.10). Given that, it is conceivable to assume that the endogenous protein may be localised both in the nucleus and in the cytoplasm of the cell, although this hypothesis still needs to be proved.

In contrast transiently expressed EGFP-tagged Poh1 localises only to the cytoplasm. Just few cells showed punctuate fluorescence in the nucleus that is possibly due to a high transient expression level of the protein. In order to overcome possible artefacts due to transient expression and also to better compare this result with the localization of the stably expressed (HA-tagged) protein, the pEGFP-POH1 plasmid was stably expressed in COS-7 cells. Unfortunately, it was impossible to obtain a population of

cells stably expressing EGFP-Poh1 fusion protein as well as EGFP protein alone. This may reflect a previously documented toxic effect of the GFP protein in mammalian cells.

The two different localization patterns of HA-Poh1 might be explained by the different epitope tags fused to the amino-terminus of the *POH1* sequence. The single HA tag in the pcDNA3 vector is only 9 amino acids long. In contrast, the 30 kDa EGFP tag leads to a great increase of the molecular weight mass of Poh1 (37 kDa). EGFP tag could modify the conformational structure of Poh1 preventing the entry of the protein into the nucleus. Poh1 does not seem to contain any consensus nuclear export or import sequences. We might assume that the protein is able to diffuse through a passive transport mechanism; however, HA-Poh1 could also enter into the nucleus by a 26S proteasome-dependent mechanism, possibly through active transport. In fact, some of the α -subunits of the 20S core proteasome contain a β -importin-like domain (Voges et al. 1999) that could mediate the transport of HA-Poh1, which is located in the lid of the regulatory particle of the complex, to the nucleus.

Poh1 and the 26S proteasome complex

Poh1 is a subunit of the 19S regulatory particle of the 26S proteasome complex (Spataro et al. 1997). Given that the overexpression of HA-Poh1 confers pleiotropic resistance to unrelated drugs, a possible role of proteolysis in determining the sensitivity of the human cells to the treatment with cytotoxic drugs has been proposed. In line with this idea, several temperature sensitive fission yeast proteasomal mutants that confer resistance to the spindle poison MBC or to a wider range of drugs have been identified over the last few years, suggesting that drug resistance might be a

result of a partial destabilisation of the 19S regulatory complex (Gordon et al. 1993; Gordon et al. 1996; Penney et al. 1998; Wilkinson et al. 1997).

HA-Poh1 has been detected in the immunopurified proteasomal preparation from lung cancer cells as well as in the purified 26S preparation from human erythrocytes (Spataro et al. 1997). Furthermore, both the fission and the budding yeast homologues *pad1⁺* and *MPR1* encode a regulatory subunit of the 26S proteasome complex (Penney et al. 1998; Rinaldi et al. 1998), indicating a high degree of conservation of this protein during evolution.

One of the main questions addressed was if the exogenous HA-Poh1 is able to confer drug resistance by a mechanism dependent on the 26S proteasome and hence presumably on the proteolytic process. Only a small amount of the exogenous Poh1 protein was associated with the 26S proteasome, whereas most of the protein was found in smaller complexes (Figure 3.10 and 3.11). In contrast, endogenous HA-Poh1 was present exclusively in the 26S proteasome-containing fractions. Nevertheless, a small amount of exogenous HA-Poh1 co-immunoprecipitated with the 20S core subunit C3 (Figure 3.12). The same result was also confirmed in human cells after inducible expression of HA-Poh1 (data not shown).

These data raise the question of which fraction of the overproduced HA-Poh1, the 26S proteasome-associated or the non-associated, underlies the pleiotropic drug resistance phenotype. HA-Poh1 not associated with the 26S proteasome complex might be able either to activate some drug resistance effectors by a 26S proteasome-independent mechanism or alternatively to disrupt some specific functions of the proteasome (see Chapter 5). Alternatively it is possible that the low amount of HA-Poh1 bound to the proteasome complex is responsible for the multi-drug resistance phenotype, which could result from specific alteration of 26S proteasome function.

Chapter 4

HA-POH1 inducible expression in human cells

Overview

In this chapter I introduce the system used to express *HA-POH1* in a doxycyclin-inducible manner in the U2-OS human osteosarcoma cell line. Inducible expression of HA-Poh1 is shown to induce pleiotropic drug resistance in human cells.

Introduction

The tetracycline/doxycyclin mammalian inducible expression system

The ability to tightly control gene expression can facilitate the analysis of the gene function. Inducible gene expression has been an advantageous tool in studying gene function in a wide variety of organisms, such as bacteria, yeast and *Drosophila melanogaster*.

The achievement of this goal in mammalian cells has been difficult due to the lack of tightness of control or to pleiotropic effects of the inducing agents, such as heat shock proteins, heavy metal ions or steroid hormones. The main objectives of an inducible system in the eukaryotic cells are a high ratio of "on:off" gene expression and the ability to vary the level of induction (Gossen and Bujard 1992).

It has been previously demonstrated that the *lac* repressor/operator/inducer system from *E. coli* is able to function in mammalian cells. Unfortunately, the use of this system has been limited as the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) acts slowly and often inefficiently leading to a moderate induction of the gene of interest (Gossen and Bujard 1992).

Gossen and Bujard described (Gossen and Bujard 1992) a control system in human HeLa cells based on the regulatory element of the Tn10-specified tetracycline (Tc) resistance operon from *E.coli*. In this system the tetracycline repressor (*tetR*) negatively regulates the transcription of the resistance-mediating gene. In bacteria, the *tetR* protein is encoded by the *tetR* gene and is tightly bound to the tetracycline operator (*tetO*) sequence located within the promoter region of the operon. This binding leads to the subsequent inhibition of expression of the genes involved in tetracycline resistance. In contrast, in the presence of the antibiotic tetracycline, *tetR* no longer binds to its operator (*tetO*) allowing gene transcription.

In order to create a protein able to stimulate a minimal promoter fused to seven repeats of the tetracycline operator sequence (*tetO*), the hybrid transactivator tTA was generated. tTA was created by combining the *tetR* sequence with the C-terminal region of the Herpes Simplex Virus (HSV) VP16 protein, which is essential for transcription of the immediate early viral genes. These synthetic promoters are silent in the presence of even low concentration of tetracycline as it prevents the binding of the tetracycline-controlled transactivator (tTA) to the *tetO* sequence, presumably through a change in the conformation of the *tetR* region in the tTA chimeric protein (Gossen and Bujard 1992) (Figure 4.1).

This system seemed to have a number of advantages over the previous described systems due to:

- The specificity of the *tetR* for its operator sequence (*tetO*)
- The high affinity of tetracycline for *tetR*
- The well-studied chemical and physiological properties of tetracycline
- The fast activity of tetracycline as an inducer

In 1995 Gossen *et al.* (Gossen et al. 1995) developed another system in which tetracycline, or the most potent tetracycline derivative doxycyclin (Dox), acts as the inducer instead of as the repressor. The reverse tetracycline-controlled transactivator (rtTA) has reversed DNA binding properties when compared with the wild-type tetR or tTA. In fact, in this system the presence of tetracycline or doxycyclin is necessary for the binding of the reverse tetracycline transactivator (rtTA) to the *tetO* sequence and hence for the subsequent activation of the expression of the gene of interest (Figure 4.2). The reversed *tTA* sequence was generated by random mutagenesis of the *tetR* sequence. The mutants were screened for the tetracycline-dependence of the repression *in vivo*. One mutant was found able to cause the repression of the gene under the tetracycline operator control in presence of the inducer, possibly due to a change of its conformation during the induction. The mutant was referred as the reverse tetracycline repressor (rtetR). The reverse tetracycline-controlled TA (rtTA) was obtained by replacing the wild-type *tetR* sequence with the mutant *rtetR* sequence in the original *tTA* hybrid DNA sequence (Gossen et al. 1995).

One main advantage of this system is its application in *in vivo* studies, as the kinetics of induction does not depend on the biological half-life of the effector (Gossen et al. 1995).

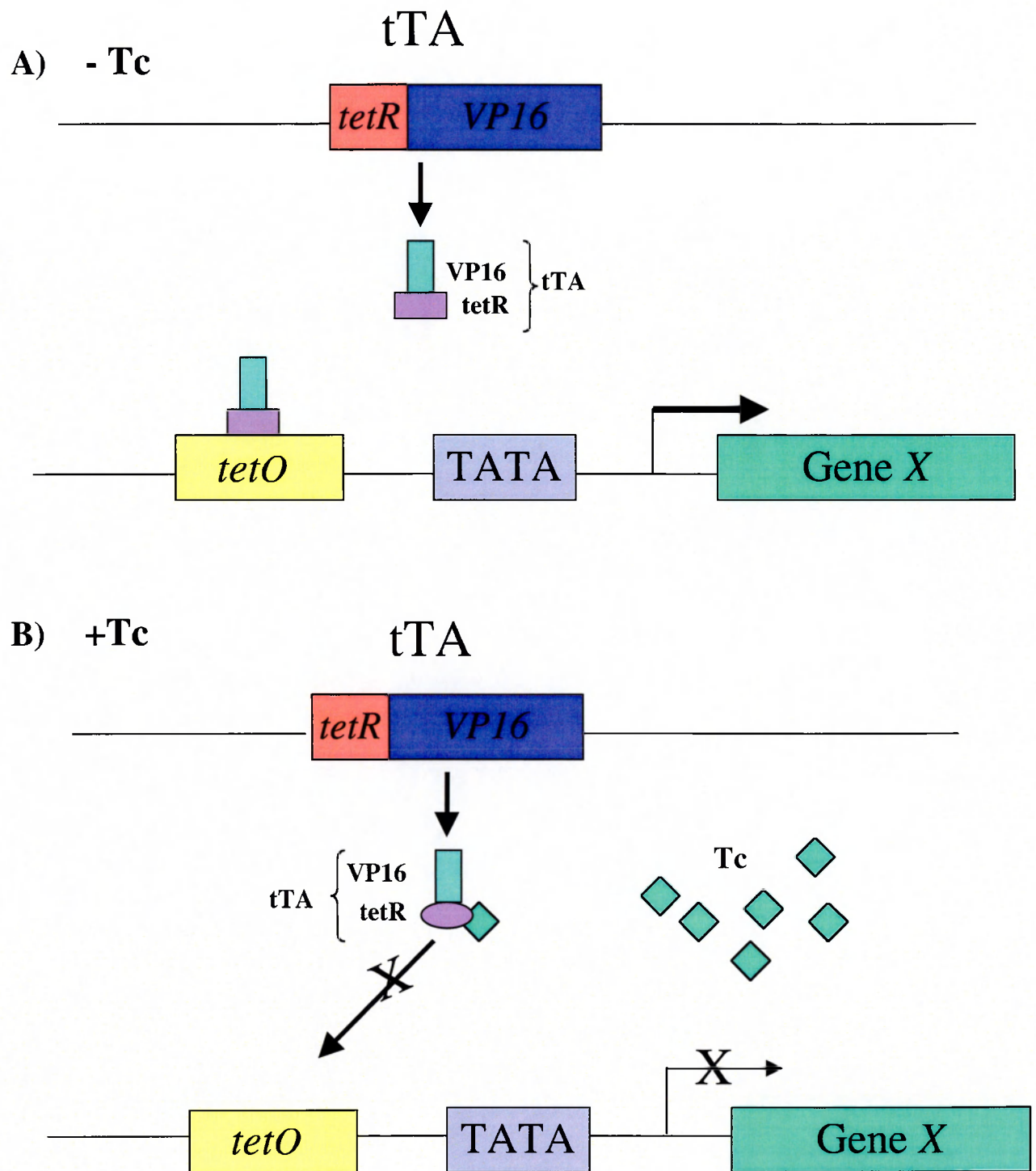


Figure 4.1 – The tetracycline inducible system

The DNA binding domain of the tetracycline (Tc) repressor (*tetR*) fused to the transcriptional activator VP16 from the herpes simplex virus (HSV) encodes the tetracycline transactivator (tTA). Tetracycline acts as repressor. A) In absence of tetracycline, the fusion protein binds to the tetracycline operator (*tetO*) through the *tetR* binding domain resulting in the activation of the expression of the gene X mediated by the VP16 activation domain. B) In presence of tetracycline, the tTA fusion protein is no longer able to bind to *tetO* due to a change of conformation with consequent repression of gene expression.

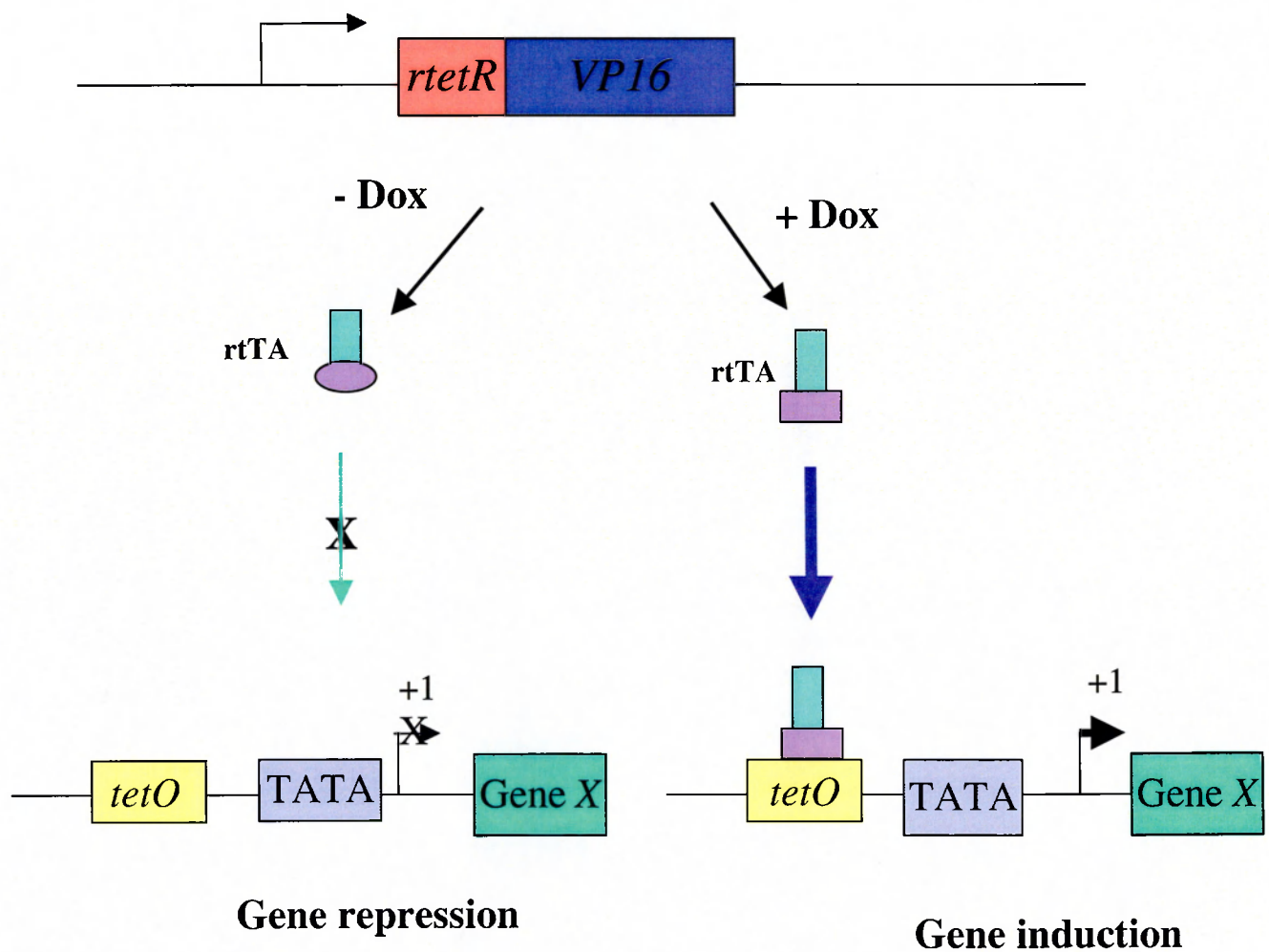


Figure 4.2 - The reverse tetracycline transactivator

In this system tetracycline (Tc) or its derivative doxycycline (Dox) act as inducer instead of as repressor. The reverse tetracycline-controlled transactivator (rtTA) has reversed DNA binding property when compared to the wild-type *tetR* or *tTA*. In presence of Tc or Dox, rtTA binds to *tetO* with subsequent activation of the expression of the gene of interest.

Results

***HA-POH1* inducible expression systems used**

The first two attempts to generate inducible expression of *HA-POH1* were unsuccessful.

- First a tetracycline repressible "Tet-off" system was used, which was based on the positive regulation of gene expression by the tetracycline transactivator (*tTA*) (Gossen and Bujard 1992). The system involved the stable transfection of two vectors. Unfortunately, it was not possible to generate regulated expression of *Poh1* with this system possibly due to technical problems with the stable selection of the cells.

- As a second approach an IPTG inducible expression system was used.

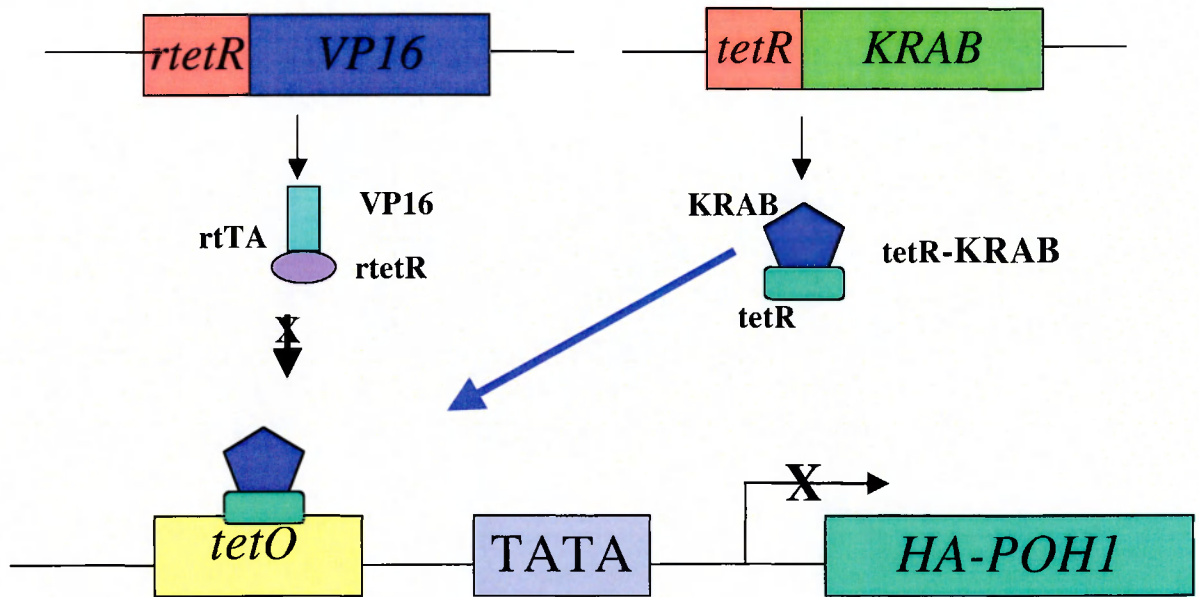
This system is based on two vectors in which several elements of the bacterial *lac* operon have been modified for use in eukaryotic cells. Human HT1080 fibrosarcoma cells were first stably transfected with the p3'SS vector, which expresses a modified *lacI* gene encoding the Lac repressor protein with a nuclear localization signal (NLS) (by S. Houlbrooke). The second vector pOPRSVI/HA-POH1 was subsequently transfected into the same cells. This vector directs the expression of the HA-tagged gene under the control of a modified Rous sarcoma virus (RSV) promoter that contains two copies of the *lac* operator sequence. The Lac repressor binds to the *lac* operator blocking the expression of the gene. When present in the medium, the isopropyl- β -D-thiogalactopyranoside (IPTG) synthetic inducer binds to Lac repressor causing a decrease of the affinity of the repressor for the operator and subsequent expression of the gene of interest.

Several stable clones expressing HA-Poh1 after induction with the IPTG [1 mM] for 24 hours were obtained (data not shown). Unfortunately, the expression level of the exogenous protein was not high. The few drug sensitivity experiments carried out on these stable clones did not show any significant difference between the induced and the un-induced populations. Using the same system identical results were also obtained for the expression of other genes under study in the laboratory.

- Finally another tetracycline/doxycyclin inducible system, which is based on a positive (activator) and a negative (repressor) regulation of gene expression, was chosen. The system contains three vectors:

1. p β rtTA expresses the reverse transactivator (rtTA) that is necessary for the transcriptional activation of the gene of interest (Gossen et al. 1995).
2. pEFblastetRKRAB expresses the repressor KRAB. In this system the inducer tetracycline (or doxycyclin) controls the silencing of the eukaryotic promoter. The KRAB repressor domain, which is a 75 amino acids sequence derived from the human Krox1 zinc finger protein, is fused to a tetR DNA binding domain (Deuschle et al. 1995). In the absence of the inducer the chimeric protein tetR-KRAB binds to several cis-acting *tetO* sites that are placed at a distance from the tetracycline initiation site of an eukaryotic promoter. When present in the medium, the inducer binds both the tetR and the rtetR domains in the recombinant proteins with their subsequent conformational change. This process inhibits the binding of tetR to the *tetO* sequence, allowing at the same time the binding of the rtetR to the *tetO* sequence and subsequent transcriptional activation of the promoter (Figure 4.3).
3. pUHD10-MCS/HA-POH1 drives the expression of the *HA-POH1* gene under the control of the CMV promoter.

A) -Dox



B) +Dox

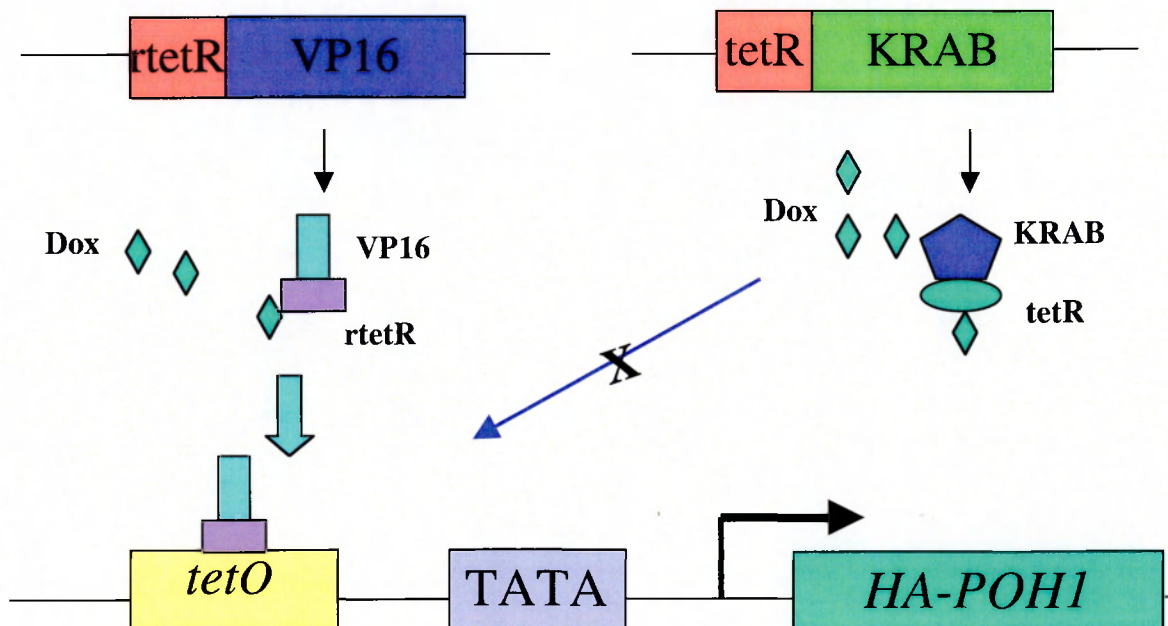


Figure 4.3 – The doxycycline inducible system for *HA-POH1* gene expression

Inducible doxycycline “on” system. A) In absence of doxycycline, the reverse transactivator rtTA is not able to bind *tetO*, whereas the repressor tetR-KRAB binds to *tetO* and inhibits gene transcription. B) In presence of doxycycline, tetR-KRAB does not binds *tetO* any longer due to a conformational change in the tetR domain. In parallel, doxycycline also induces a change in the rtetR domain of the rtTA with the consequent binding of rtTA to *tetO* and activation of the transcription of *HA-POH1*.

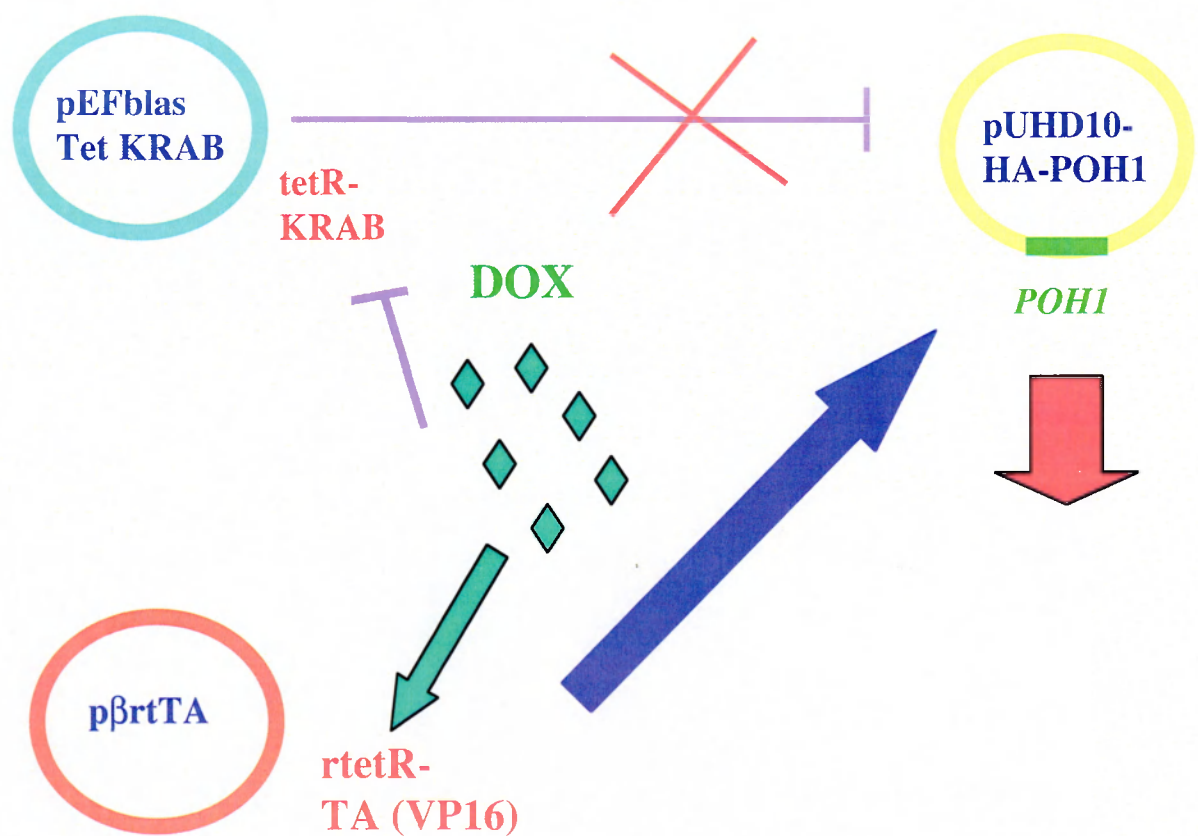


Figure 4.4 – Regulation of the inducible HA-Poh1 expression

A simplified diagram of the HA-Poh1 inducible system is shown in Figure 4.4.

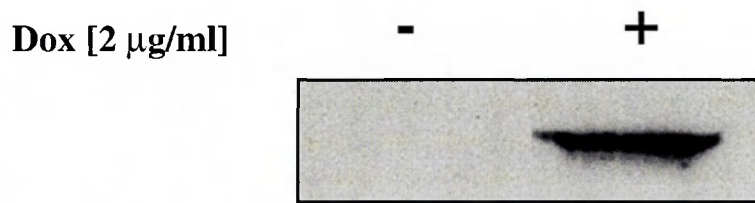
Inducible expression of HA-Poh1 in human cells

A human U2-OS osteosarcoma stable clone co-transfected with p β *prtTA* and pEFblast*etRKRAB* vectors and selected using the antibiotic blasticidin S [5 μ g/ml] was generously provided by Dr. S.Geley (ICRF Clare Hall). The U2-OS *prtTA/ptetRKRAB* cells were then co-transfected with pUHD10-MCS/HA-POH1 and pcDNA3.1+ (ratio 4:1), as carrier of the G-418 resistance marker for selection of transfected cells. After two weeks of selection with G-418, the stable polyclonal cells were tested for the expression of HA-Poh1 after the induction with doxycyclin [3 μ g/ml] for 16 hours. Protein detection was performed by immunoblot analysis on whole cell lysates using HRP-conjugated monoclonal anti-HA antibody (Figure 4.5A). In parallel, 60-70 stable clones were screened for the expression of HA-Poh1 after induction with doxycyclin. In total, 5 positive clones were found. Figure 3.5B shows the expression of HA-Poh1 in some of the stable clones as detected by immunoblot analysis.

It has been previously described that doxycyclin at concentration as low as 10 ng/ml is able to induce gene expression using this system, while the concentration of 1 μ g/ml has been suggested to be optimal for gene induction (Gossen et al. 1995).

In order to find the optimal concentration of doxycyclin for the expression of HA-Poh1 in U2-OS cells a dose response experiment was carried out. Cells from the polyclonal pool population were exposed to several concentrations of doxycyclin (0.025, 0.1, 0.5 and 1.5 μ g/ml) or no doxycyclin (Figure 4.6A). The results show that the concentration of 25 ng/ml was already able to induce HA-Poh1 expression and, in addition, HA-Poh1 expression level was dose-dependent. Figure 4.6 panel B

A) Polyclonal pool



B) Stable clones

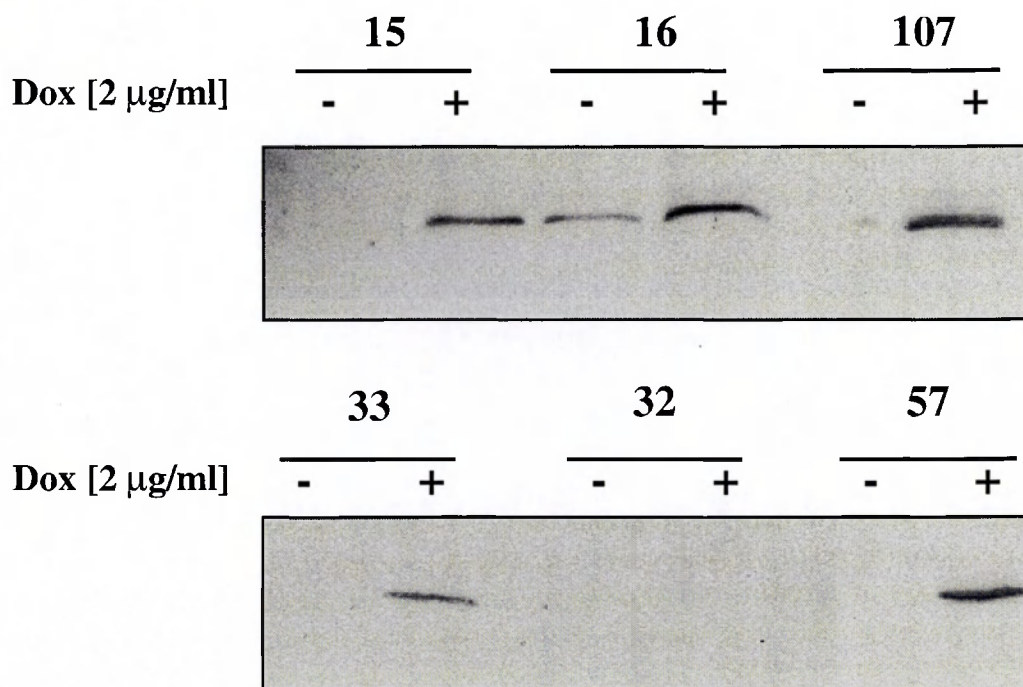


Figure 4.5 - Doxycyclin inducible expression of HA-Poh1 in stably transfected U2-OS cells

Immunoblot analysis of HA-Poh1 induction in the human U2-OS cells. The cells from polyclonal pool (A) and some of stable clones (B) were mock (-) or doxycyclin (+) [2 μ g/ml] treated for 16 hours. Whole cell lysates were subjected to immunoblot analysis using HRP-conjugated monoclonal anti-HA antibody.

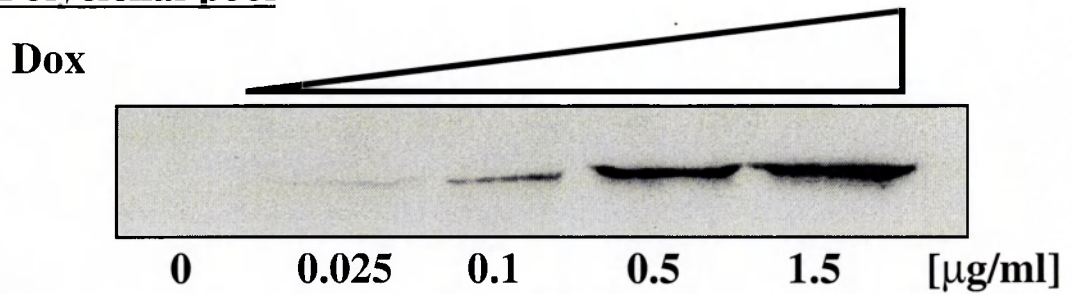
shows the inducible expression of HA-Poh1 in the stable clone 33 (C33) cells using a range of doxycyclin from 0.1 to 2.5 $\mu\text{g/ml}$. Taken together these results suggest that doxycyclin concentrations between 0.5 and 1.5 $\mu\text{g/ml}$ are optimal for the inducible expression of HA-Poh1; a concentration between 1-1.5 $\mu\text{g/ml}$ was used in most subsequent experiments.

Next, cells from the two stable clones C15 and C33 were mock or doxycyclin treated [1.5 $\mu\text{g/ml}$] for 24, 36 and 48 hours and the time-dependency of HA-Poh1 expression was studied (Figure 4.7A). Doxycyclin has a half-life of 24 hours, so every day [0.75 $\mu\text{g/ml}$] doxycyclin was added in order to maintain its final concentration of 1.5 $\mu\text{g/ml}$. The protein level of HA-Poh1 was fairly constant under these conditions and did not change significantly between 24 and 48 hours. Furthermore, treatment of the cells from clone 33 with doxycyclin [1 $\mu\text{g/ml}$] for 8 days led to a constant expression of HA-Poh1 throughout the time (Figure 4.7B). Cdc2 protein was detected as loading control in the same cell lysates by using monoclonal anti-Cdc2 antibody (Figure 4.7, lower panel).

No toxic effects of doxycyclin were observed in all these experiments, even after 8 days of treatment.

Exogenous HA-Poh1 was detected in both the soluble and the nuclear enriched fractions of the stably transfected COS-7 cells after cell fractionation (Figure 3.8). This result was also observed in human cells. Cells from clone 33 were mock or doxycyclin [1 $\mu\text{g/ml}$] treated for 24 hours and the nuclear and cytoplasmic fractions were separated as already described for COS-7 cells in chapter 3 and analysed by immunoblotting. Figure 4.8 shows the expression of both the overexpressed and endogenous HA-Poh1 in both fractions.

A) Polyclonal pool



B) C 33

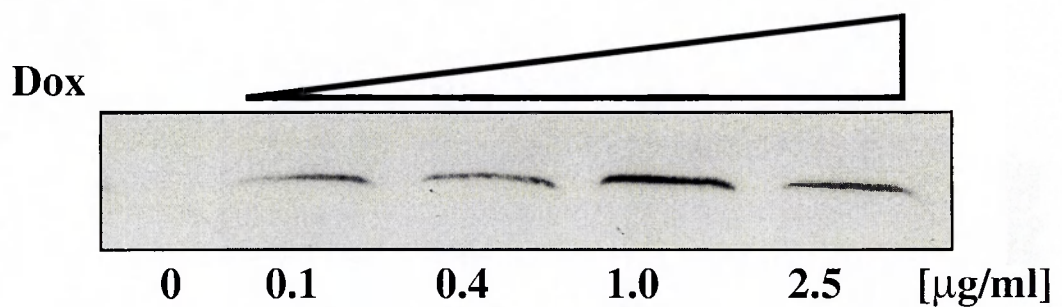


Figure 4.6 - Doxycyclin dose response curve of HA-Poh1 expression in U2-OS cells
HA-Poh1 expression was induced or not with different concentrations of doxycyclin for 16 hours in cells from stable polyclonal pool (A) and clone 33 (B). Whole cell lysates were analysed by immunoblotting using HRP-conjugated monoclonal anti-HA antibody.

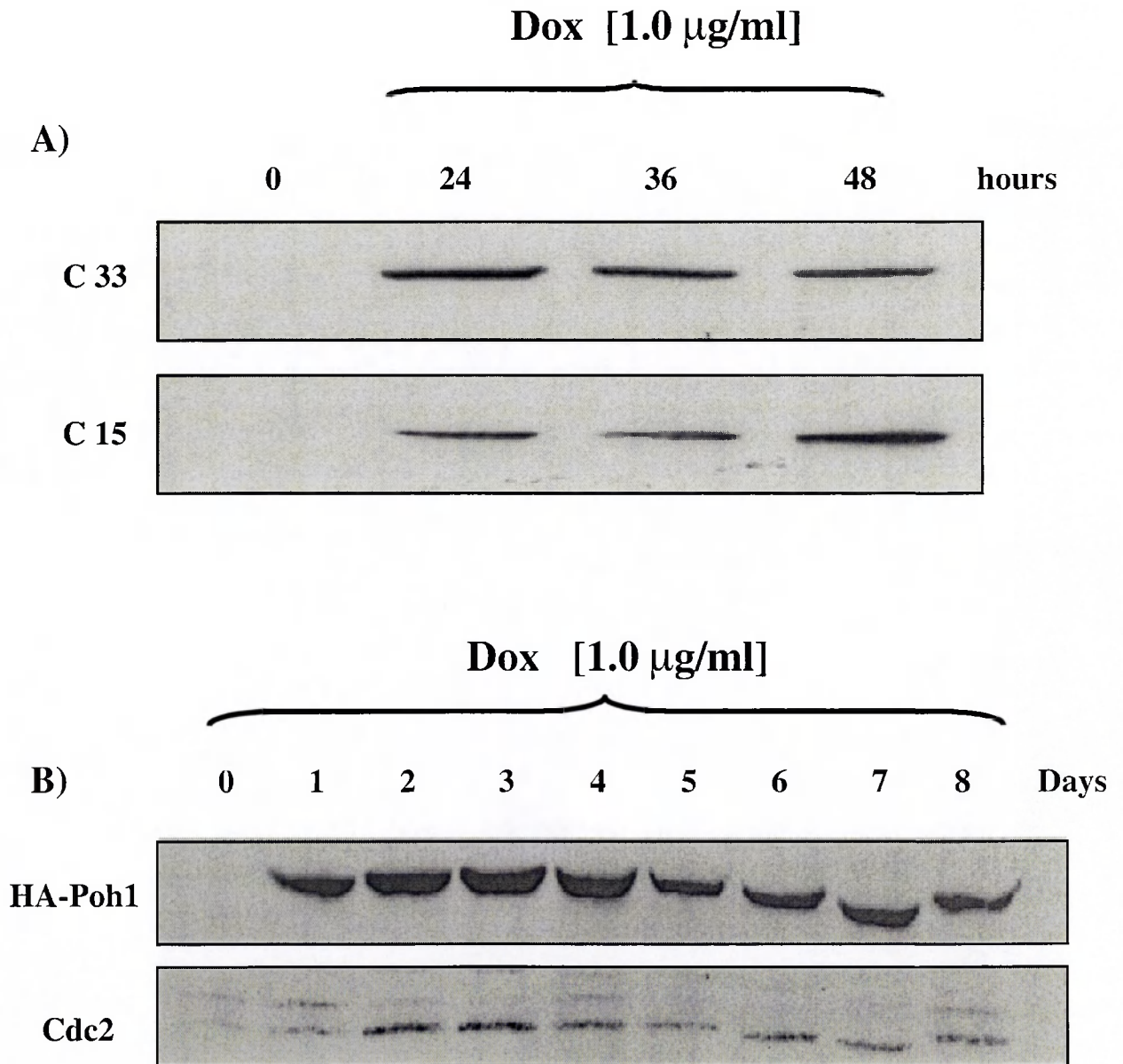


Figure 4.7 - Time course of HA-Poh1 expression in doxycyclin inducible stable clones
 Detection of induced HA-Poh1 by immunoblot analysis. Cells from U2-OS stable clones 15 and 33 were mock or doxycyclin treated for 0, 24, 36 and 48 hours with 1.5 µg/ml (A) and cells from clone 33 for 0 to 8 days with 1.0 µg/ml (B). Doxycyclin [0.75 µg/ml] or [0.5 µg/ml] was added to the cells every 24 hours. Whole cell lysates were analysed by immunoblotting using HRP-conjugated monoclonal anti-HA antibody. As loading control Cdc2 was detected using mouse monoclonal anti-Cdc2 (A17) antibody.

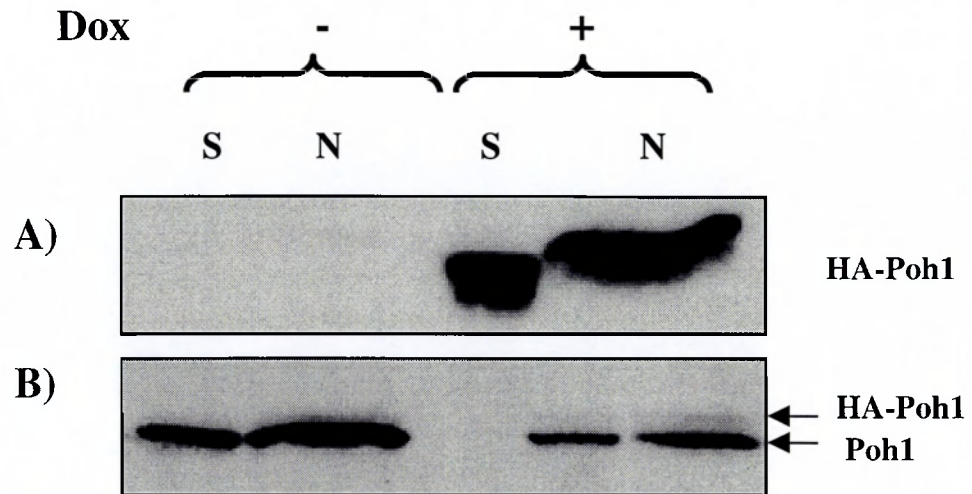


Figure 4.8 – Endogenous and exogenous Poh1 are expressed both in the soluble and in the nuclear enriched fractions of human cells

Detection of HA-Poh1 and Poh1 by immunoblot analysis in nuclear and soluble fractions from mock or doxycycline treated U2-OS cells (clone 33). A) Exogenous Poh1 was detected using HRP-conjugated monoclonal anti-HA antibody, whereas B) endogenous Poh1 was detected using rabbit polyclonal anti-Poh1 CN13 antibody.

Inducible expression of HA-Poh1 confers drug resistance in human cells

Transient expression of *HA-POH1* in COS-1 cells leads to a multi-drug resistance phenotype (Spataro et al. 1997). This result was supported by the stable expression of the protein in COS-7 cells, as described in chapter 3. Before further investigating the role of HA-Poh1 overexpression in mammalian and human cells (Chapter 5), the ability of inducible HA-Poh1 to confer drug resistance in human cells was tested.

First, in order to exclude possible effects of doxycyclin treatment on the sensitivity of the cells to cytotoxic drugs, the U2-OS polyclonal cells carrying the empty vector pUHD10-MCS were mock or doxycyclin [1 µg/ml] treated for 24 hours. Then, the two cell populations were exposed to several dilutions of melphalan (Figure 4.9A) or 7-hydroxystaurosporine (Figure 4.9B) for 72 hours. The viability of the cells was detected using the MTS cytotoxicity assay. No effect of doxycyclin treatment on the susceptibility of the cells to other drugs was found.

Next the sensitivity of HA-Poh1 transfected cells was investigated. Cells from clones 15 and 57 were mock or doxycyclin treated for 24 hours and clone 15 cells were treated with 7-hydroxystaurosporine (Figure 4.10B) and clone 57 cells were treated with 7-hydroxystaurosporine, melphalan or doxorubicin (Figure 4.10A, C, D) for 72 hours. Cell viability was measured using the MTS cytotoxicity assay. These results together with those shown in Chapter 3 establish that expression of HA-Poh1 in human cells leads to significant resistance to a variety of unrelated drugs, as previously observed in mammalian and fission yeast cells (Spataro et al. 1997). The higher resistance to doxorubicin observed in human cells in figure 4.10D in respect to the one observed in COS-7 cells using the clonogenic assay (Chapter 3, Figure 3.8) is possibly due to the different approaches used.

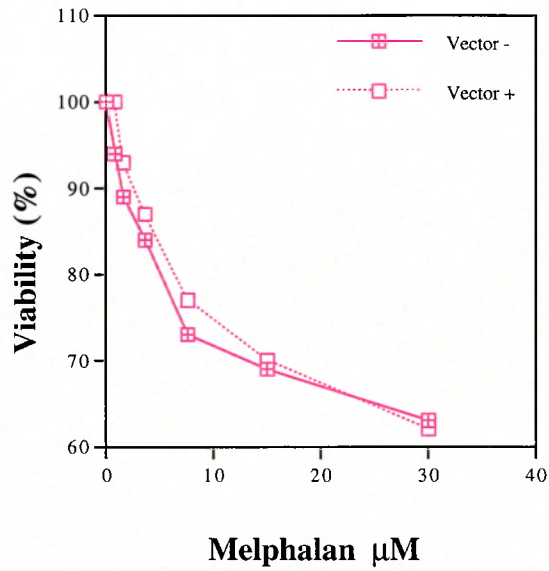
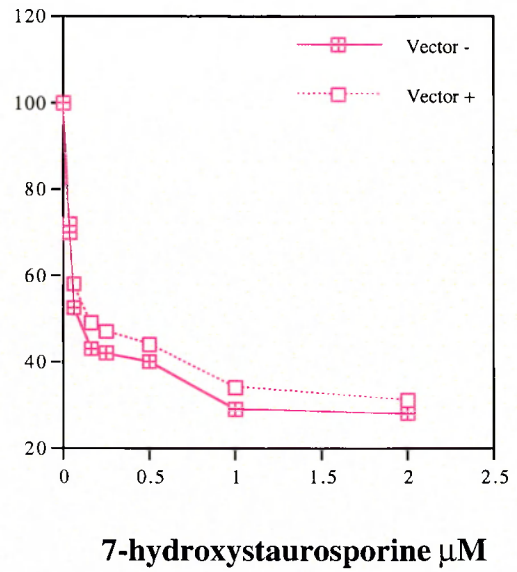
A)**B)**

Figure 4.9 – Treatment with doxycycline does not affect the susceptibility of U2-OS cells to other drugs

MTS cytotoxicity assay in U2-OS cells carrying the empty vector, mock (-) or doxycycline (+) treated. 1000 cells were plated in triplicate in a 96-multiwell plate. 24 hours later the cells were treated with melphalan (A) or 7-hydroxystaurosporine (B) for 72 hours. Cell viability was determined by MTS assay. Results are expressed as the percentage viability compared to the control (no drug) cells.

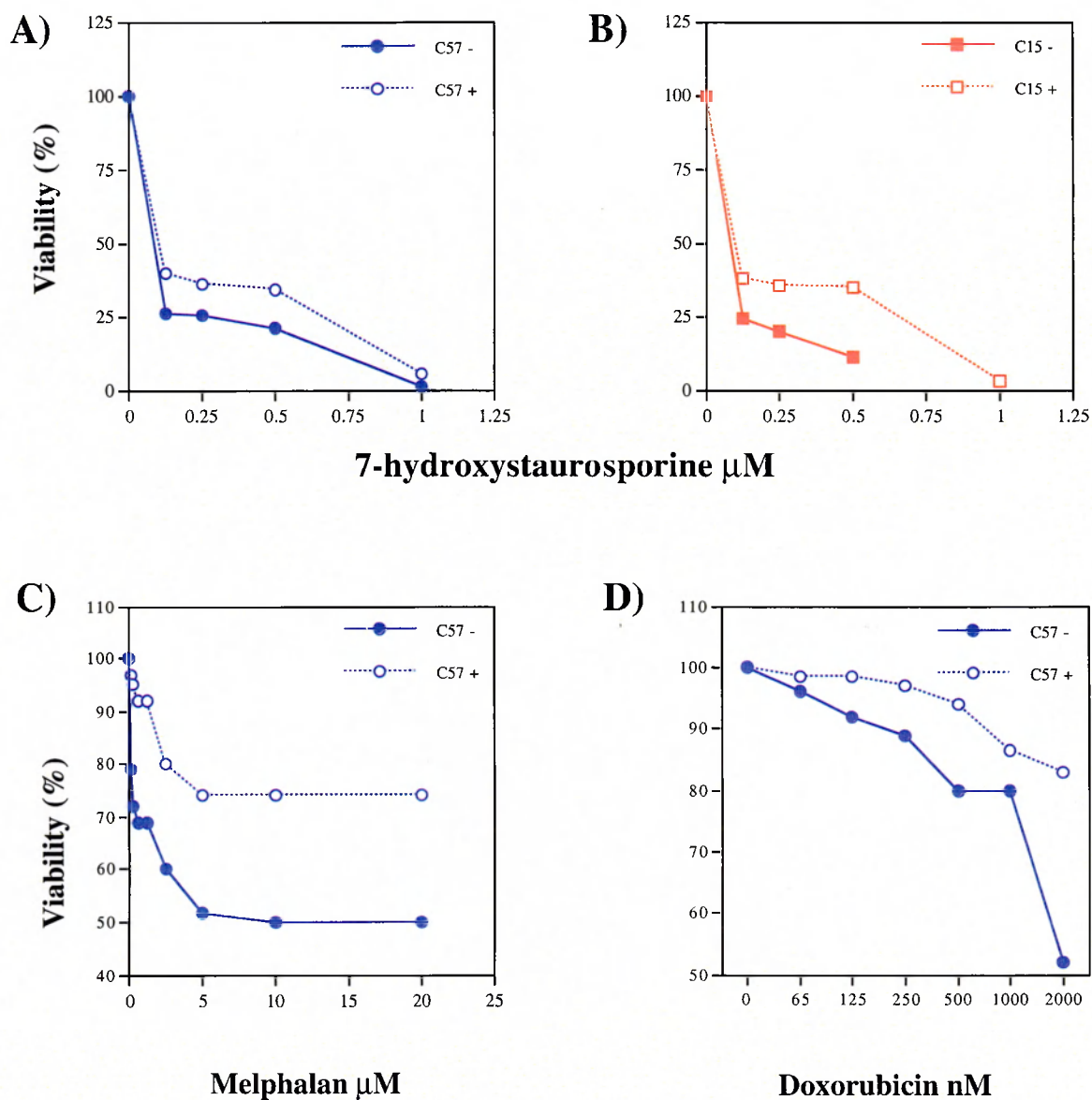


Figure 4.10 - Inducible expression of HA-Poh1 confers pleiotropic resistance in human cells

MTS cytotoxic assay in U2-OS cells transfected with HA-Poh1, mock (-) or doxycyclin (+) treated. 1000 cells were plated in triplicate in a 96-multiwell plate. 24 hours later the cells from clone 57 (A) and clone 15 (B) were treated with 7-hydroxystaurosporine. Cells from clone 57 were treated with melphalan (C) or doxorubicin (D) for 72 hours. Cell viability was determined using the MTS assay. Results are expressed as the percentage viability compared to the control cells (no drug).

Chapter 5

HA-Poh1 and AP-1 transcription factors

Overview

In this chapter I describe the molecular mechanism by which overexpressed HA-Poh1 leads to pleiotropic drug resistance in mammalian cells.

Firstly, I introduce AP-1 transcription factors, the links between AP-1 and the 26S proteasome complex and drug resistance in human cells. Secondly, I describe the ability of HA-Poh1 to positively modulate AP-1 transcription factor activity associated with increased protein levels of the two AP-1 subunits, c-Jun and c-Fos. Furthermore, evidence is presented that HA-Poh1 may increase the protein stability of c-Jun through an interaction between the two proteins.

Introduction

Activating protein 1 (AP-1)

Activating protein 1 (AP-1) is a family of dimeric transcription factors complex, whose components include c-Jun and c-Fos, members of the bZip family proteins which contain conserved basic (b) and leucine zipper (ZIP) domains required for their binding to DNA. This motif mediates homodimerization of c-Jun and heterodimerization of c-Jun and c-Fos and the subsequent recognition and binding to the palindromic consensus DNA sequence 5'-TGAGTCA-3', referred as 12-*O*-Tetradecanoylphorbol 13-acetate (TPA)-responsive element (TRE) or AP-1 consensus site, located in the promoter region of a variety of genes (Angel et al. 1987b). c-Jun and c-Fos are both defined proto-oncogenes as they were identified as the cellular counterparts of the oncogenes v-Jun and v-Fos, respectively (Maki

et al. 1987) (Curran and Teich 1982). c-Jun is also able to form heterodimeric complexes with ATF-2 (Activating Transcription Factor 2), belonging to the ATF transcription factor family, and CREB proteins. The resulting complexes bind to the AP-1-related cyclic AMP responsive element (CRE)-like site 5'-TGAGCTCA-3' (reviewed by Karin et al. 1997; Wisdom 1999).

A wide range of external stimuli, such as peptide growth factors, cytokines, oxidative stress, UV irradiation and other forms of cellular stress, generate signals that converge on the AP-1 factors (Figure 5.1), with subsequent modulation of their activity which is regulated primarily at the transcriptional and post-translational levels (Figure 5.2). Based on this regulation, AP-1 co-ordinates a variety of signals and consequently generates diverse cellular responses, including cell proliferation, differentiation, development, apoptosis and other stress responses (reviewed by Karin et al. 1997; Wisdom 1999) (Figure 5.1).

DNA binding and transcriptional activation domains are distinct in c-Jun and c-Fos and the activation domains are modulated by phosphorylation. For example, c-Jun is phosphorylated at Ser⁶³, Ser⁷³, Thr⁹¹ and Thr⁹³ by members of the c-Jun NH₂-terminal kinases (JNK) family (Adler et al. 1994). JNK, originally identified as a c-Jun associated kinase, is a serine/threonine kinase activated by phosphorylation by various stimuli, including DNA damaging agents, inhibitors of protein synthesis, heat shock and osmotic stresses (reviewed by Kyriakis et al. 1994). Phosphorylation of c-Jun controls its activity in two manners (Figure 5.2). On the one hand, the tight association between JNK and c-Jun, which is independent on the activation status of JNK, targets c-Jun for ubiquitinylation and on the other hand phosphorylation at Ser⁷³ by JNK is sufficient to protect the protein from ubiquitinylation resulting in increased protein stabilization (Fuchs et al. 1997) (Figure 5.3). This probably occurs through a conformational change of c-Jun after phosphorylation which consequently affects its affinity for JNK.

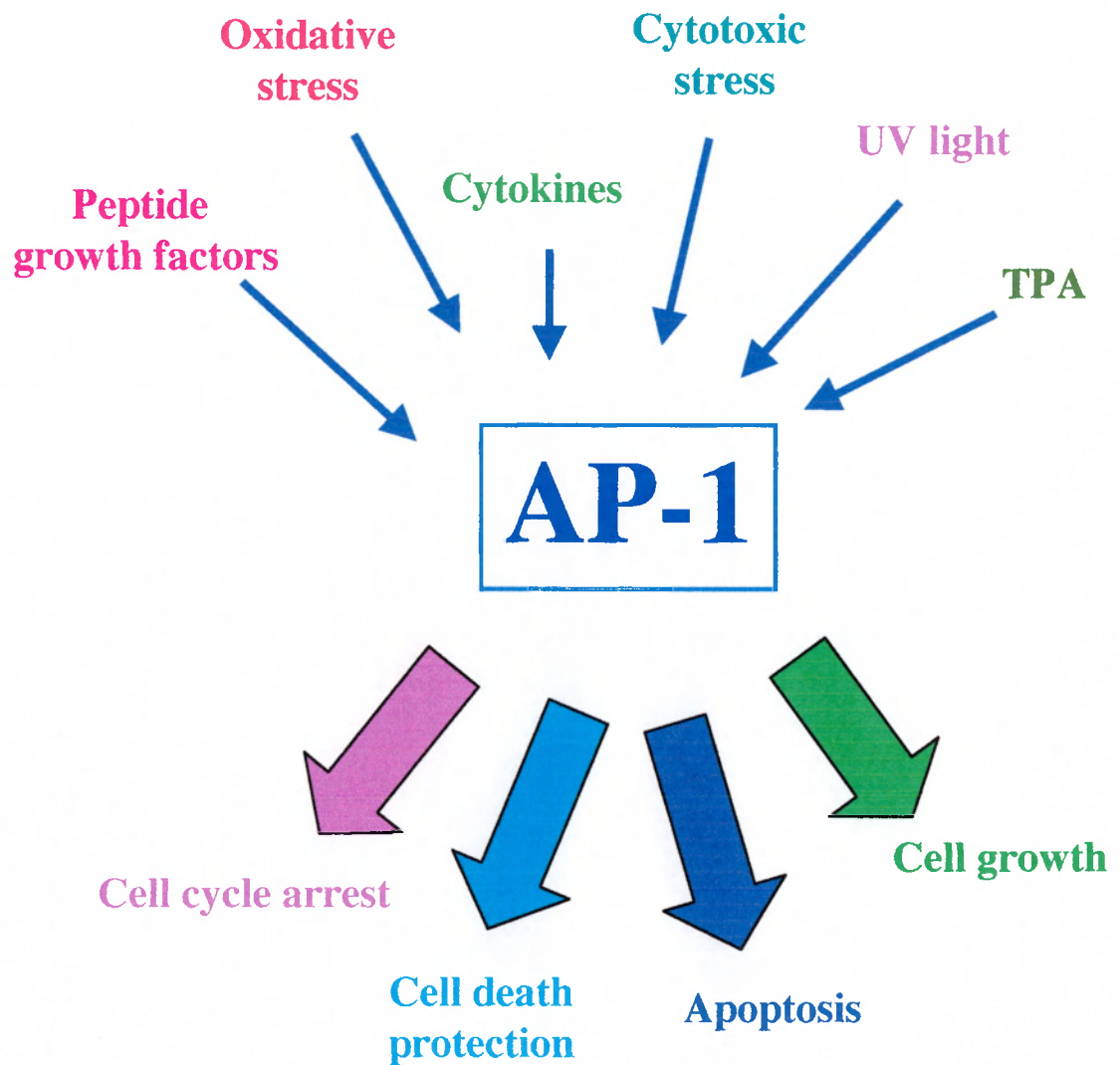


Figure 5.1 – AP-1 transcription factors: stimuli and responses

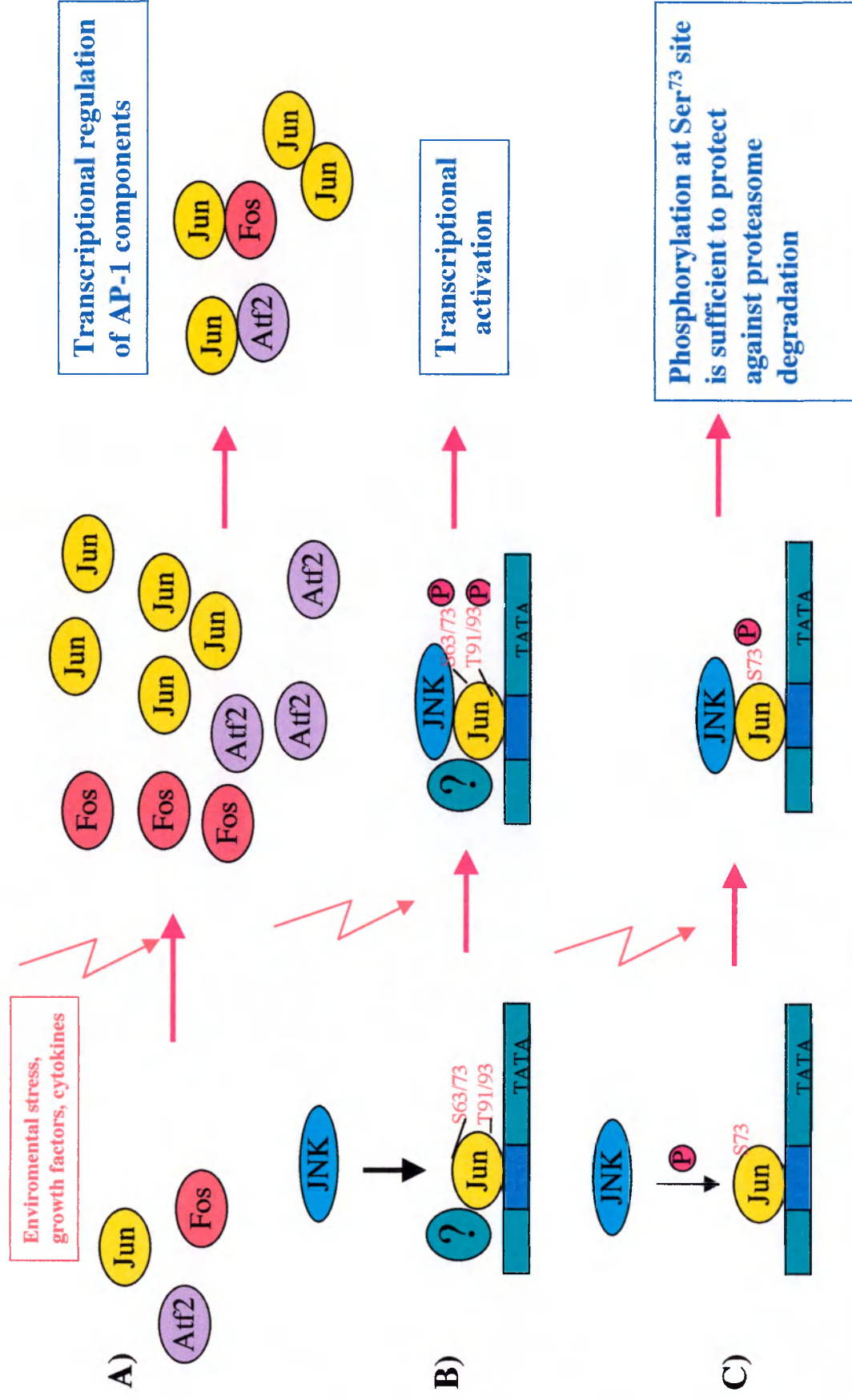


Figure 5.2 – Regulation of c-Jun activity

c-Jun can be regulated at the transcriptional level (A) and at the post-translational level (B, C) by a wide variety of external stimuli. A) Gene expression of c-Jun and its association partners is increased with subsequent elevated level of the respective proteins and dimerization. B) c-Jun is phosphorylated by JNK resulting in transcriptional activation. C) Phosphorylation at Ser⁷³ by JNK is sufficient to protect c-Jun from ubiquitin-dependent proteolysis, resulting in stabilization of the protein.

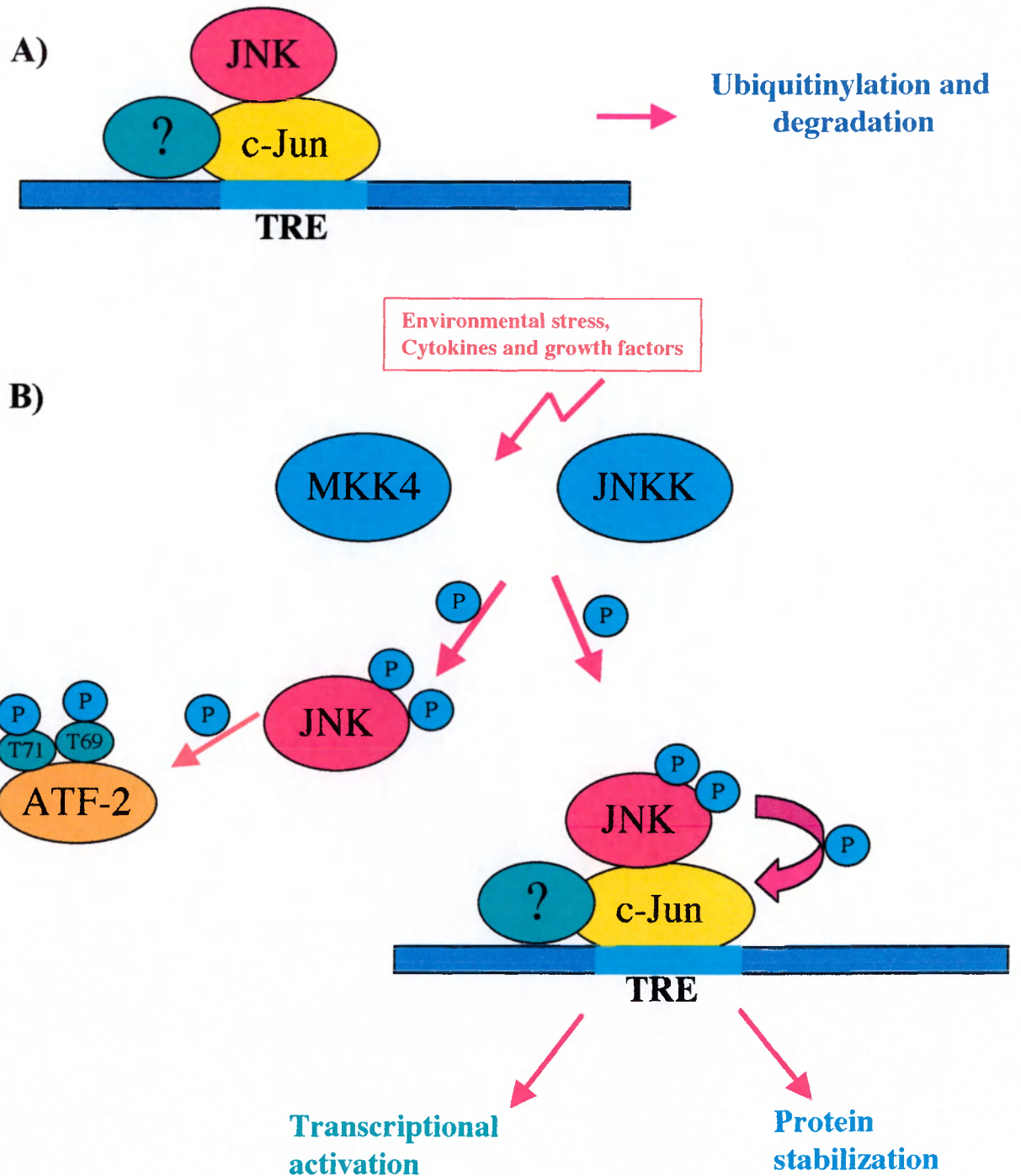


Figure 5.3 – c-Jun regulation by JNK

A) JNK tightly binds to the δ domain of c-Jun independently on its activation status. This association leads to ubiquitinylation of c-Jun and its subsequently degradation. B) After stress, JNK kinase, activated by phosphorylation via the MAP kinase pathway, phosphorylates c-Jun at serine and threonine phosphoacceptor sites. c-Jun is in turn activated at the transcriptional level and stabilized.

A few lines of evidence have demonstrated that in fibroblasts the mitogenic response of the cell is mediated through AP-1 activity, resulting in modulation of cell growth and proliferation (Brown et al. 1998; Kovary and Bravo 1991; Maki et al. 1987). c-Jun and c-Fos play also a major role in the cellular response to UV irradiation by protecting the cell from UV-induced cell death (Wisdom, 1999) (Schreiber et al. 1995). It was recently reported that c-Jun modulates cell proliferation and UV responses by distinct mechanisms, both involving modulation of the transcription of the cyclin D1 gene (Wisdom, 1999) (Wisdom et al. 1999). Regulation of cell proliferation does not require c-Jun phosphorylation, whereas phosphorylation of c-Jun at Ser⁶³ and Ser⁷³ sites with subsequent activation of the protein is necessary to protect the cell from UV-induced cell death.

c-Jun degradation pathways

c-Jun is a short-lived protein with a half-life of approximately 90 minutes (Treier et al. 1994). Tight temporal control of the activity of many regulators of gene transcription, including c-Fos, p53, c-Mos and c-Myc, shares this feature (Rogers et al. 1986).

c-Jun and c-Fos are both degraded by the 26S proteasome complex (Treier et al. 1994; Papavassiliou et al. 1992; Ciechanover et al. 1991). However, several aspects of the regulation of AP-1 turnover are controversial, perhaps suggesting the participation of multiple pathways. Treier *et al.* showed that c-Jun is ubiquitinated and degraded by the 26S proteasome pathway *in vivo* (Treier et al. 1994) and in addition that the δ domain, a 27 amino acid sequence located at NH₂-terminus of the protein, is essential for c-Jun ubiquitination and its subsequent proteolysis. In line with these findings, the oncogenic counterpart v-Jun, which lacks the δ domain due to an in frame-deletion, was shown to have elevated protein stability in comparison to c-Jun (Treier et al. 1994). JNK binds to the δ domain of c-Jun and consequently targets its substrate for ubiquitination (Fuchs et al.

1996). In contrast, *in vitro* studies of AP-1 ubiquitinylation using reconstituted enzyme systems showed that c-Jun lacking the δ domain and c-Fos lacking the PEST motif, a common sequence involved in the rapid destruction of short-lived proteins (Rogers et al. 1986), can be efficiently ubiquitinated (Hermida-Matsumoto et al. 1996). It was also reported that degradation of c-Jun by the 26S proteasome pathway does not require ubiquitinylation of the protein *in vitro* (Jariel-Encontre et al. 1995). Another number of substrates, including ornithine decarboxylase, is degraded by the 26S proteasome in a ubiquitin-independent manner. c-Jun might contain intrinsic signals for ubiquitin-independent recognition by the 26S proteasome complex and subsequent degradation. In addition, a role for calpains (calcium-dependent cysteine proteases) in the degradation of c-Jun and c-Fos has been shown. However, these proteases may have only limited involvement in AP-1 turnover, as they are restricted to the cytoplasm (Jariel-Encontre et al. 1995).

Taken together, these data imply that the regulation of c-Jun and c-Fos turnover is complex and controversial but also establish that the major proteolytic pathway for their regulation involves the 26S proteasome pathway. Whether ubiquitinylation is an absolute requirement for the proteolysis *in vivo* of these proteins, and whether different pathways co-exist, possibly depending on the physiological conditions of the cell or the cell type, are still open issues.

One interesting general observation that has recently come to light is the inverse correlation between the stability of transcriptional activators and their activation domain potency *in vivo* (Molinari et al. 1999). It seems that the rate of degradation of transcription factors by the 26S proteasome pathway strongly depends on the degree of their activity, as highly

potent transcription activators are processed very rapidly in mammalian cells. In order to be degraded these activators must interact with their targets.

AP-1 and drug resistance

Some studies have suggested a role for AP-1 factors in the modulation of drug resistance in cancer cell lines. Multi-drug resistance MCF-7 breast cancer cells exhibited elevated expression of *c-jun* and *c-fos* genes together with elevated transcriptional and DNA binding activities of AP-1 (Daschner et al. 1999). AP-1 modulation of the *MDR-1* gene, which contains a putative AP-1 binding site in its promoter region with consequent increase of MDR-1 mRNA and protein level have also been documented. Furthermore, c-Jun mRNA level and DNA binding activity of AP-1 were also reported to be elevated in etoposide resistant human leukemia cells (Ritke et al. 1994). Other reports have shown a positive correlation between AP-1 activation and glutathione-S-transferase levels in drug resistant cancer cell lines (Moffat et al. 1994; Puchalski and Fahl 1990).

Results

HA-Poh1 increases the activity of AP-1 transcription factors

The main question addressed in this chapter is by which molecular mechanism exogenously overexpressed HA-Poh1 confers multi-drug resistance in mammalian cells. Genetic studies previously carried out in fission yeast showed that the drug resistance phenotype induced by HA-Poh1 in *S.pombe* (like that of fission yeast Pad1) is dependent on the AP-1-like transcription factor Pap1 (Spataro et al. 1997).

To this end, the ability of overexpressed HA-Poh1 to modulate the activity of the AP-1 transcription factors in mammalian cells was studied using two different approaches. The ability of AP-1 transcription factors to bind to its consensus DNA sequence was studied using an electrophoretic mobility shift assay, whereas transcriptional activity of AP-1 was measured using an AP-1-regulated promoter in a luciferase reporter assay.

To measure the DNA binding activity of AP-1, whole cell extracts (WCE) were prepared from polyclonal COS-7 cells stably expressing HA-Poh1 or carrying the empty vector. As a positive control for AP-1 DNA binding, cells carrying the empty vector were also treated with the tumour promoter phorbol-12-myristate 13-acetate (PMA) for 6 hours. Phorbol esters are activators of protein kinase C and also potent inducers of AP-1 activity (Angel et al. 1987b). Equal amounts of whole cell extracts from both cell populations were incubated for 30 minutes at room temperature with a 21-bp [³²P]-labelled double stranded oligonucleotide (Figure A.1 - *Appendix*), which contains the 8-bp AP-1 consensus DNA sequence 5' - TGAGTCAG - 3'. DNA/protein complexes were electrophoretically separated from free oligonucleotides under non-denaturing conditions. A more intense band

correspondent to DNA/AP-1 complex appeared in PMA-treated and HA-Poh1-overexpressing cells in comparison with PMA-untreated cells carrying the empty vector (Figure 5.4A), demonstrating up-regulated binding of AP-1 to its cognate DNA sequence. As discussed above, AP-1 activity is regulated at both the transcriptional and the post-translational level. To investigate at which level the AP-1 DNA binding activity was modulated, the protein level of c-Jun, the main component of AP-1, was detected in whole cell extracts by immunoblot analysis using a rabbit polyclonal anti-c-Jun antibody (Figure 5.4B). In line with the elevated AP-1 DNA binding activity observed in these cells, approximately 20-fold and 3-fold increases of c-Jun protein level were observed in PMA-treated cells and HA-Poh1-overexpressing cells, respectively (Figure 5.4C). Detection of Cdc2 protein was also performed as a loading control using a monoclonal anti-Cdc2 (A17) antibody (Figure 5.4B lower panel).

Given that c-Jun and c-Fos are nuclear transcription factors, AP-1 DNA binding activity was next assayed using nuclear extracts from polyclonal COS-7 cells expressing HA-Poh1 or carrying the empty vector (Figure 5.5). For competition experiments, nuclear extracts were also pre-incubated with a 50-fold excess of unlabelled ("cold") AP-1 consensus or mutant oligonucleotides, in which the consensus nucleotides CA are changed in TC (Figure A.1 - *Appendix*), for 20 minutes before adding the labelled probe (Figure 5.5A). In accord with the previous result, cells expressing exogenous HA-Poh1 showed an approximately 4-fold increase in AP-1 DNA binding in comparison with cells carrying the empty vector. This experiment also established that the DNA/AP-1 complex is AP-1 specific, as when an excess of unlabelled consensus probe was added to the binding reaction as

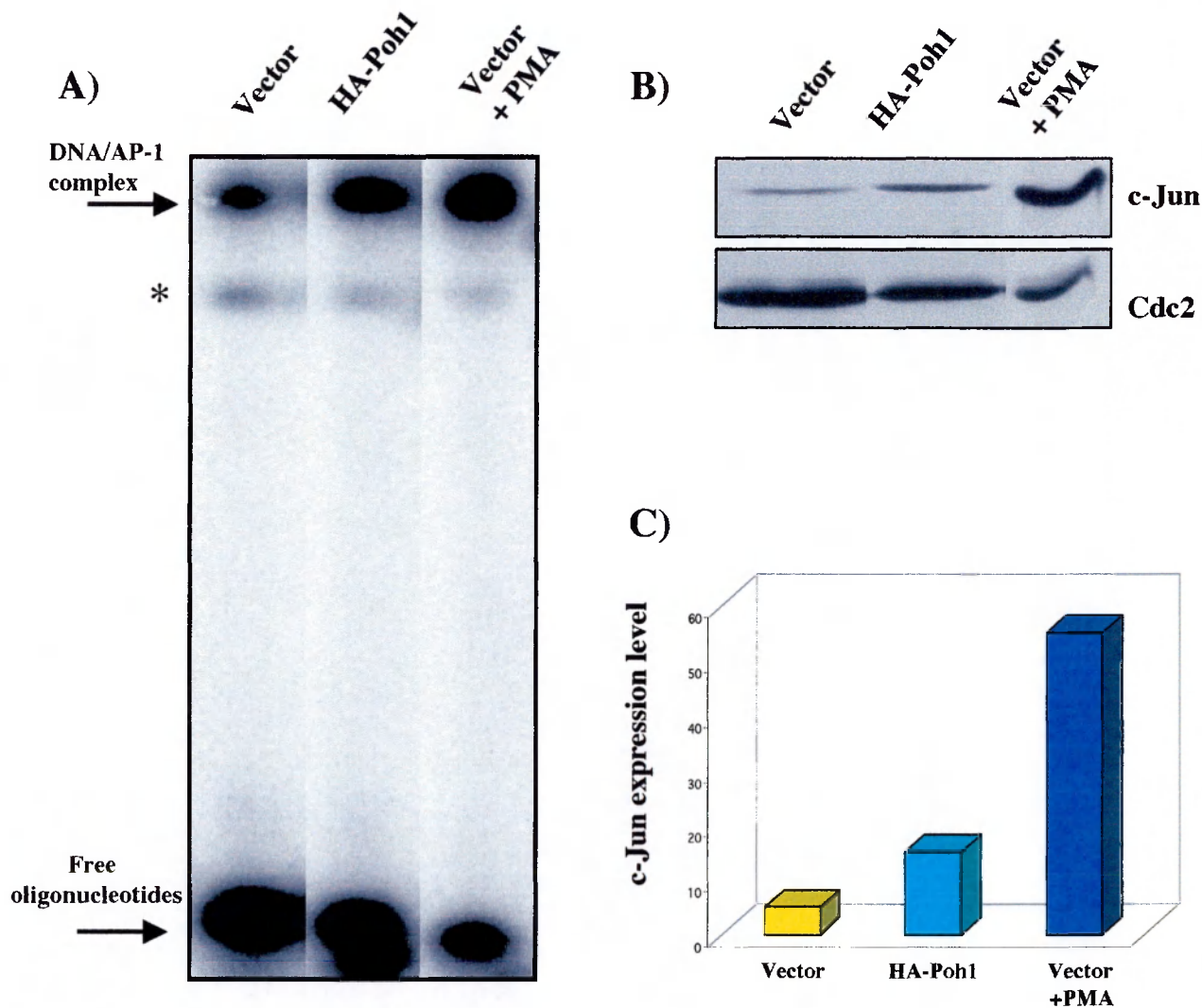


Figure 5.4 - HA-Poh1 overexpression leads to increased AP-1 binding activity and c-Jun protein level in COS-7 cells

An electrophoretic mobility shift assay was performed using whole cell extracts (WCE) prepared from polyclonal populations stably transfected with empty vector (pcDNA3.1+), mock or PMA (6 hours) treated, or pcDNA3/HA-POH1 vector. B) Immunoblot analysis of c-Jun protein level in WCE as in A using rabbit polyclonal anti-c-Jun antibody. Cdc2 protein was detected as loading control using mouse monoclonal anti-Cdc2 antibody. C) Densitometric analysis of c-Jun protein level from panel B. * non-specific band

* Non-specific band

competitor, the binding of AP-1 to its DNA sequence was abolished. In contrast the presence of an excess of unlabelled AP-1 mutant oligonucleotide did not affect the binding reaction. Additionally, 2.3- and 3.5-fold increases of c-Jun and c-Fos protein levels were observed by immunoblot analysis using rabbit polyclonal antibodies against c-Jun and Fos, respectively (Figure 5.5B and C). Detection of Hap1 DNA repair endonuclease in the same nuclear extracts was also performed as loading control (Figure 5.5B).

These results were further supported by the positive modulation at AP-1 DNA binding activity and protein level observed after doxycyclin inducible expression of HA-Poh1 in human U2-OS cells (Figure 5.6 A and B).

Next, the possible effect of overexpressed HA-Poh1 on AP-1 transcriptional activity was investigated using an AP-1-dependent luciferase reporter assay. This assay is based on transient co-transfection of the reporter and internal control plasmids. The reporter pColluc3 (kindly provided by Dr. J.Tavare) drives the expression of firefly luciferase under the control of the collagenase promoter, which contains the TPA-responsive element (TRE) and is strongly modulated by AP-1 transcription factors (Angel et al. 1987b). The pRL-TK-*Renilla* plasmid, constitutively expressing *Renilla* luciferase from the thymidine kinase (TK) gene promoter, was used as internal control for transfection efficiency (Figure 5.7). Firefly and *Renilla* luciferases have different evolutionary origin and use different substrates allowing a sequential measurement of their activities from a single sample. COS-7 cells carrying the empty vector or overexpressing HA-Poh1 (polyclonal pool, clone 2 and clone 25) (Figure 5.8A) and inducible human U2-OS cells (clones 15, 33 and 57), mock or doxycyclin treated for 24 hours (Figure 5.8B), were transiently co-

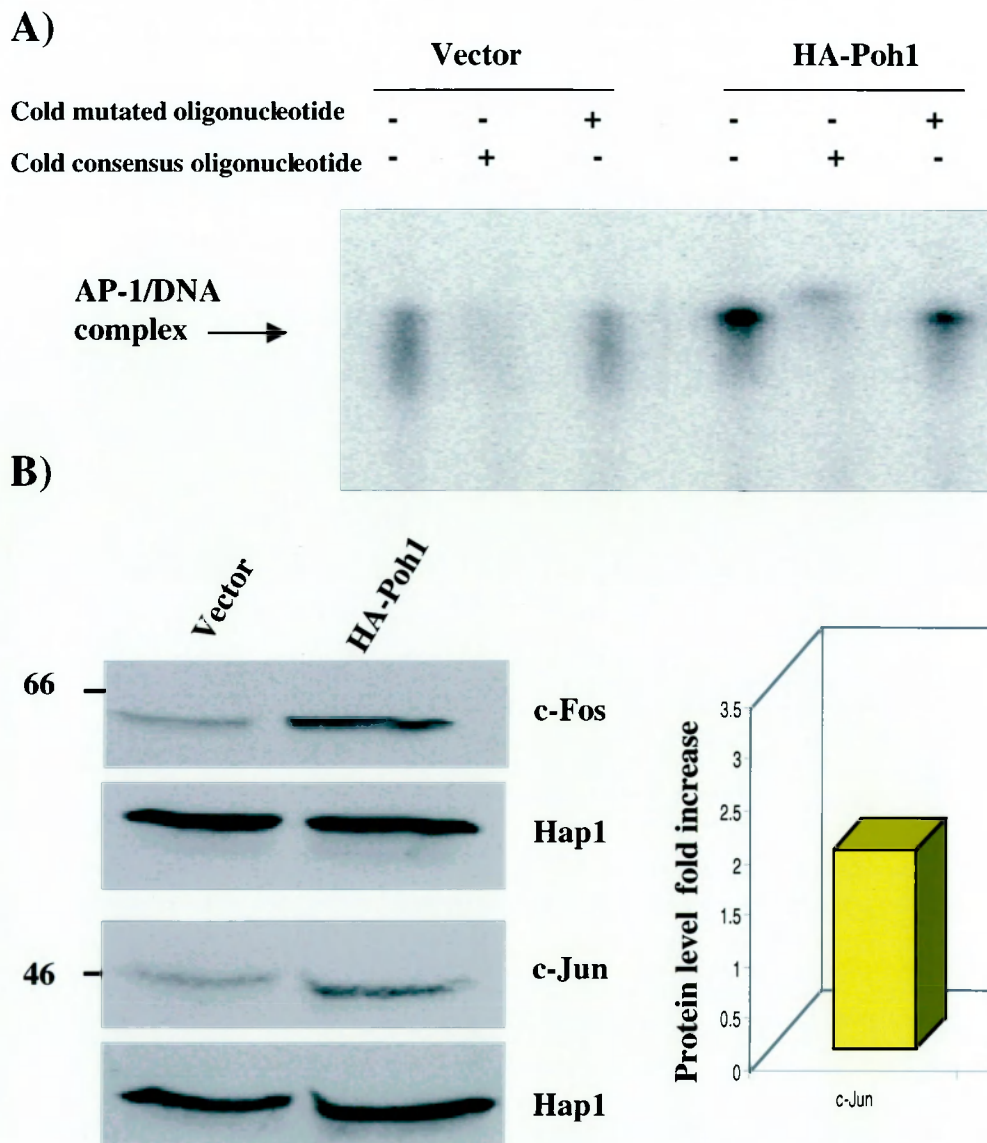


Figure 5.5 - HA-Poh1 overexpression leads to up-regulation of both AP-1 binding activity and c-Jun and c-Fos protein levels in COS7 cells

A) Electrophoretic mobility shift assay using nuclear extracts (NE) prepared from COS7 polyclonal cells stably transfected with empty vector (pcDNA3.1+) or pcDNA3/HA-POH1. 5 µg of NE were incubated with 0.5 ng of ³²P-labelled AP-1 consensus oligonucleotide for 30 minutes at room temperature. For competition experiments, a 50-fold excess of unlabelled AP-1 consensus or mutated oligonucleotides were pre-incubated with NE at room temperature 20 minutes before adding the labelled probe. B) Immunoblot analysis of c-Jun and c-Fos protein levels in NE (60 µg). Detection of c-Jun and c-Fos was carried out using rabbit polyclonal anti-c-Jun and anti-c-Fos antibodies, respectively. As a loading control DNA repair endonuclease Hap1 was detected. C) Densitometric analysis of c-Jun and c-Fos protein levels from panel B.

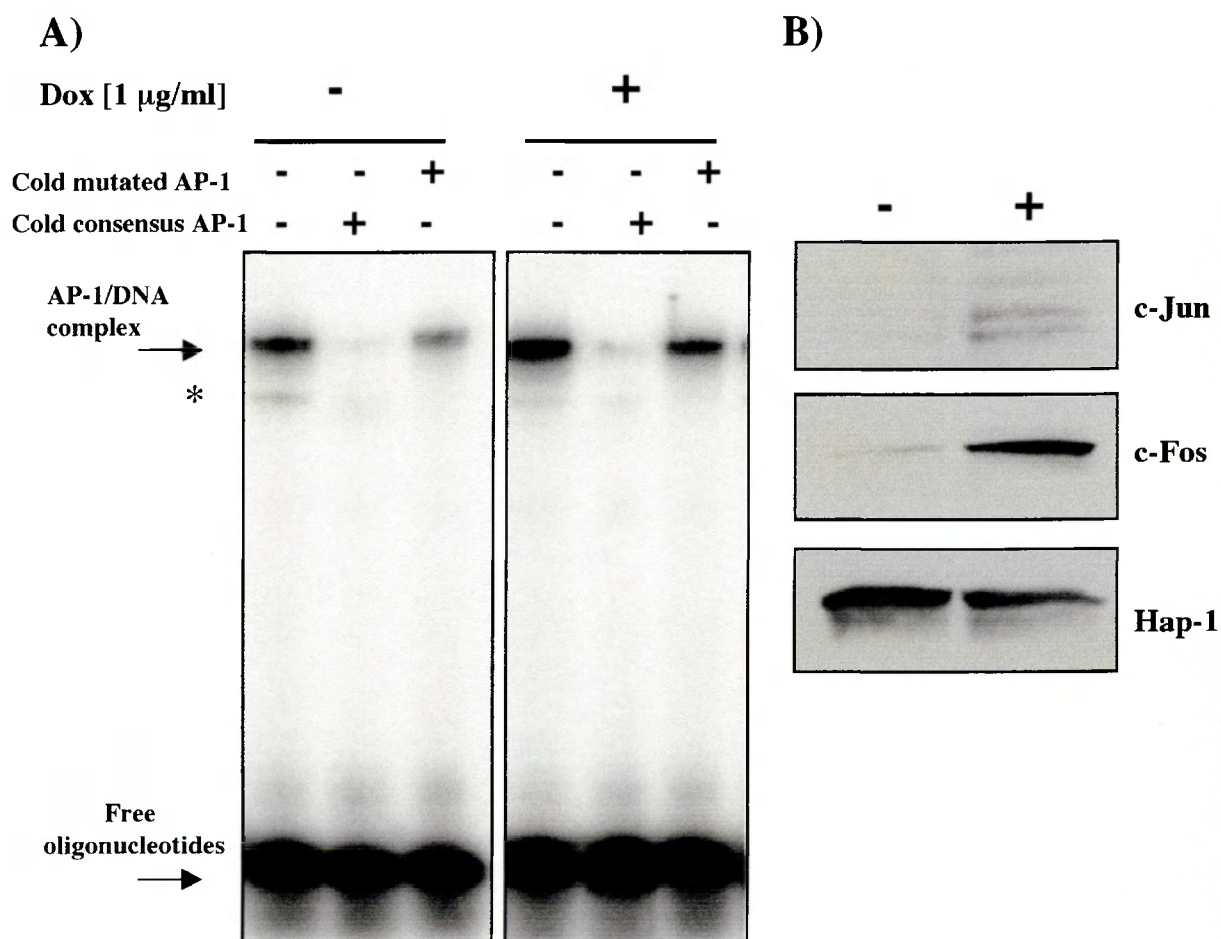


Figure 5.6- Inducible expression of HA-Poh1 up-regulates AP-1 protein levels and DNA binding activity in human cells

An electrophoretic mobility shift assay was performed using nuclear extracts (NE) prepared from U2-OS clone 33 cells, mock (-) or doxycyclin (+) treated. For competition experiments 50-fold excess of unlabelled AP-1 consensus or mutated oligonucleotides was used. B) Detection of c-Jun and c-Fos protein level in nuclear extracts (40 µg) from U2-OS clone 33 cells, mock or doxycyclin treated, by immunoblot analysis. As loading control DNA repair Hap1 protein was detected.

* Non-specific band

transfected with the reporter and control plasmids at a ratio of 15:1. 48 hours later the cells were lysed and assayed for both luciferase activities using the dual luciferase reporter kit. The results, expressed as fold-increase of AP-1-dependent relative luciferase activity (RLA) (the ratio between firefly and *Renilla* activities) (Figure 5.8) were not striking but always consistent with an average of 2-fold increase in AP-1 transcriptional activity after either constitutive (Figure 5.8A) or inducible overexpression of HA-Poh1 in mammalian cells (Figure 5.8B).

It is noteworthy that COS-7 cells from clone 2, which previously showed a better expression level of exogenous HA-Poh1 and a greater survival advantage in response to drug treatment in comparison to other HA-Poh1-overexpressing populations (Chapter 3), here also exhibited the highest AP-1-dependent luciferase activity (6-fold increase) (Figure 5.8A), as confirmed in two independent experiments.

Taken together, these data demonstrate that exogenously expressed HA-Poh1 positively modulates DNA binding and transcriptional activities of AP-1 transcription factors in mammalian cells.

Ubiquitinylation of AP-1 in HA-Poh1 expressing cells

Both c-Jun and c-Fos are ubiquitinated and degraded by the 26S proteasome pathway (Ciechanover et al. 1991; Papavassiliou et al. 1992; Stancovski et al. 1995; Treier et al. 1994). Since Poh1 is a regulatory subunit of the 26S proteasome complex (Spataro et al. 1997), involved in ubiquitin-dependent protein degradation, it was of interest to determine whether up-regulation of AP-1 activity is caused via modulation of c-Jun and c-Fos ubiquitinylation in HA-Poh1 overexpressing cells.

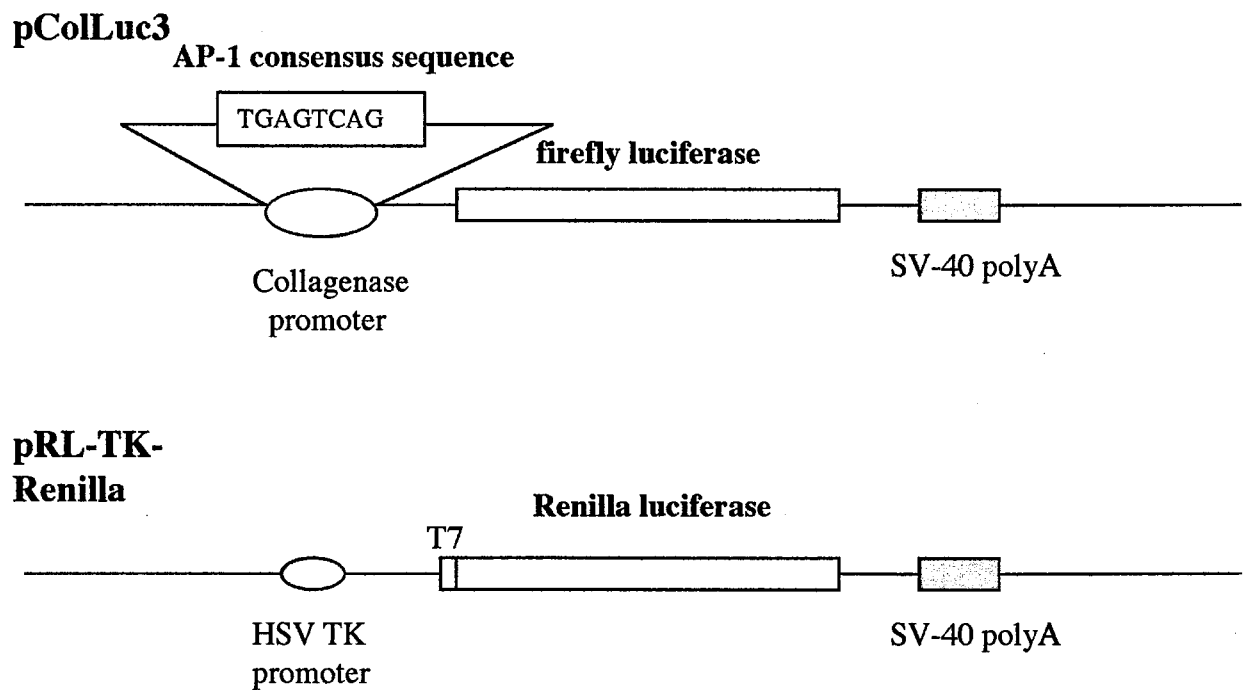
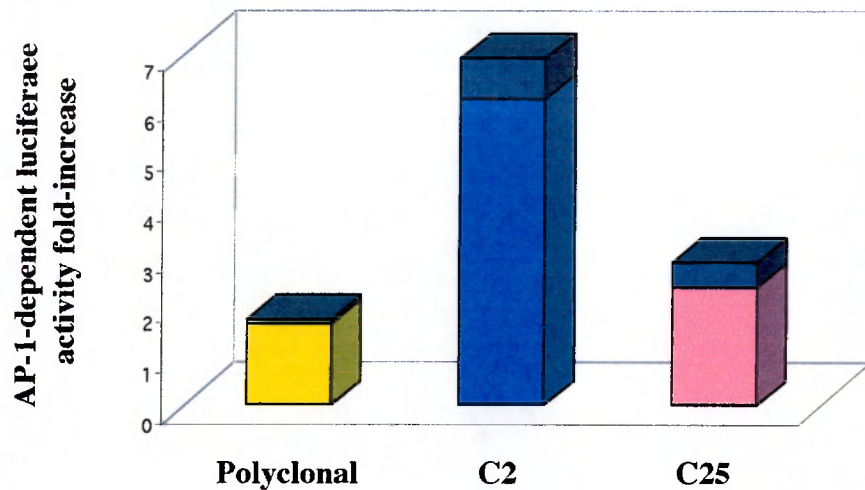


Figure 5.7- Constructs used for AP-1-dependent luciferase reporter assay

pColluc3 vector drives the expression of firefly luciferase under the control of the collagenase promoter, which contains the 9-bp consensus AP-1 responsive element TGAGTCA. pRL-TK-*Renilla*, in which the promoter from the thymidine kinase (TK) gene drives constitutive expression of *Renilla* luciferase, is used as an internal control of transfection

A)



B)

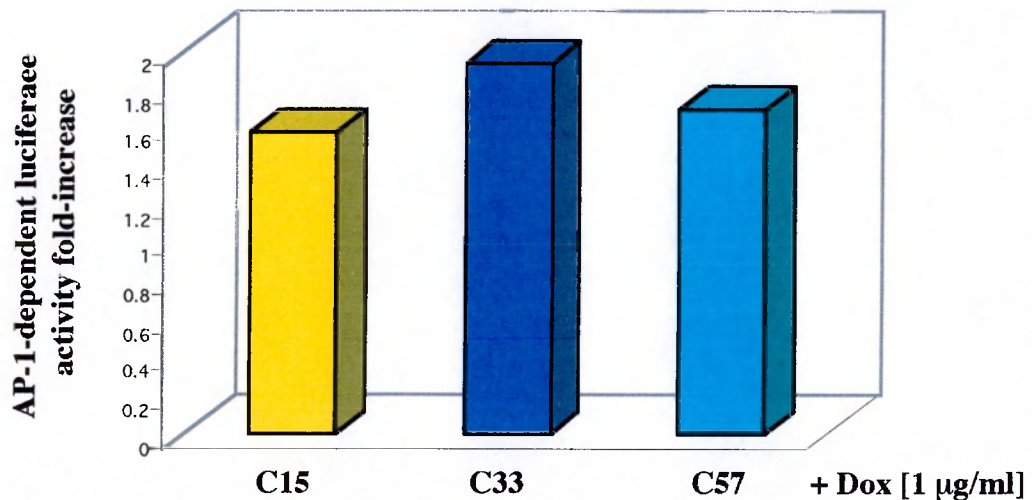


Figure 5.8 - HA-Poh1 overexpression increases AP-1 transcriptional activity

Luciferase reporter assay for AP-1 transcriptional activity. COS-7 cells from the polyclonal pool and stable clones 2 and 25 expressing HA-Poh1 or cells carrying the vector alone (A) and U2-OS inducible clones mock or doxycyclin treated (B) were co-transfected with pColluc3 and the pRL-TK-*Renilla* plasmids at a ratio of 15:1. Firefly and *Renilla* luciferase activities were measured using the dual Luciferase Reporter Kit (Promega). Results are expressed as fold-increase of the relative luciferase activity (the ratio between the firefly and the *Renilla* activities) in cells overexpressing HA-Poh1 in comparison with cells carrying the empty vector (A) or doxycyclin un-treated (B). Standard deviation is indicated by the dark blue shading in (A).

First, total protein ubiquitinylation was measured in polyclonal COS-7 cells carrying the empty vector or overexpressing HA-Poh1. Cells were lysed in 2x SDS sample buffer and protein ubiquitinylation was analyzed by immunoblot using polyclonal anti-ubiquitin antibody. HA-Poh1 overexpression did not have any effect on total protein ubiquitinylation (Figure 5.9). This experiment further demonstrated that ubiquitinylation of total proteins was inefficiently detected by the anti-ubiquitin antibody used. In line with this the detection of ubiquitinylated c-Jun and c-Fos attempted after immunoprecipitation was unsuccessful.

To overcome this technical problem, pcDNA1/HA-Ub plasmid (kindly provided by Dr. N.Masson) expressing HA-tagged Ubiquitin was used. Human U2-OS cells from clone 57, mock or doxycyclin treated for 24 hours, were transiently transfected with pcDNA/HA-Ub. As a control of transfection efficiency, equal numbers of cells were lysed in 2x SDS sample buffer and HA-ubiquitin was detected by immunoblot analysis using HRP-conjugated monoclonal anti-HA antibody (Figure 5.10A). c-Jun was then immunoprecipitated in parallel from whole cell lysates using a rabbit polyclonal anti-c-Jun antibody, and c-Jun ubiquitinylation and expression were both detected by immunoblot analysis using HRP-conjugated monoclonal anti-HA and rabbit polyclonal anti-c-Jun antibodies, respectively. The results indicate that overexpression of HA-Poh1 does not affect the general level of c-Jun ubiquitinylation in human cells, although a band of about 60 kDa that might correspond to tetraubiquitinylated c-Jun was reduced in cells treated with doxycyclin (Figure 5.10A and B).

The experiment was repeated in order to detect ubiquitinylated forms of both c-Jun and c-Fos after their immunoprecipitation (Figure 5.11). A lower level of

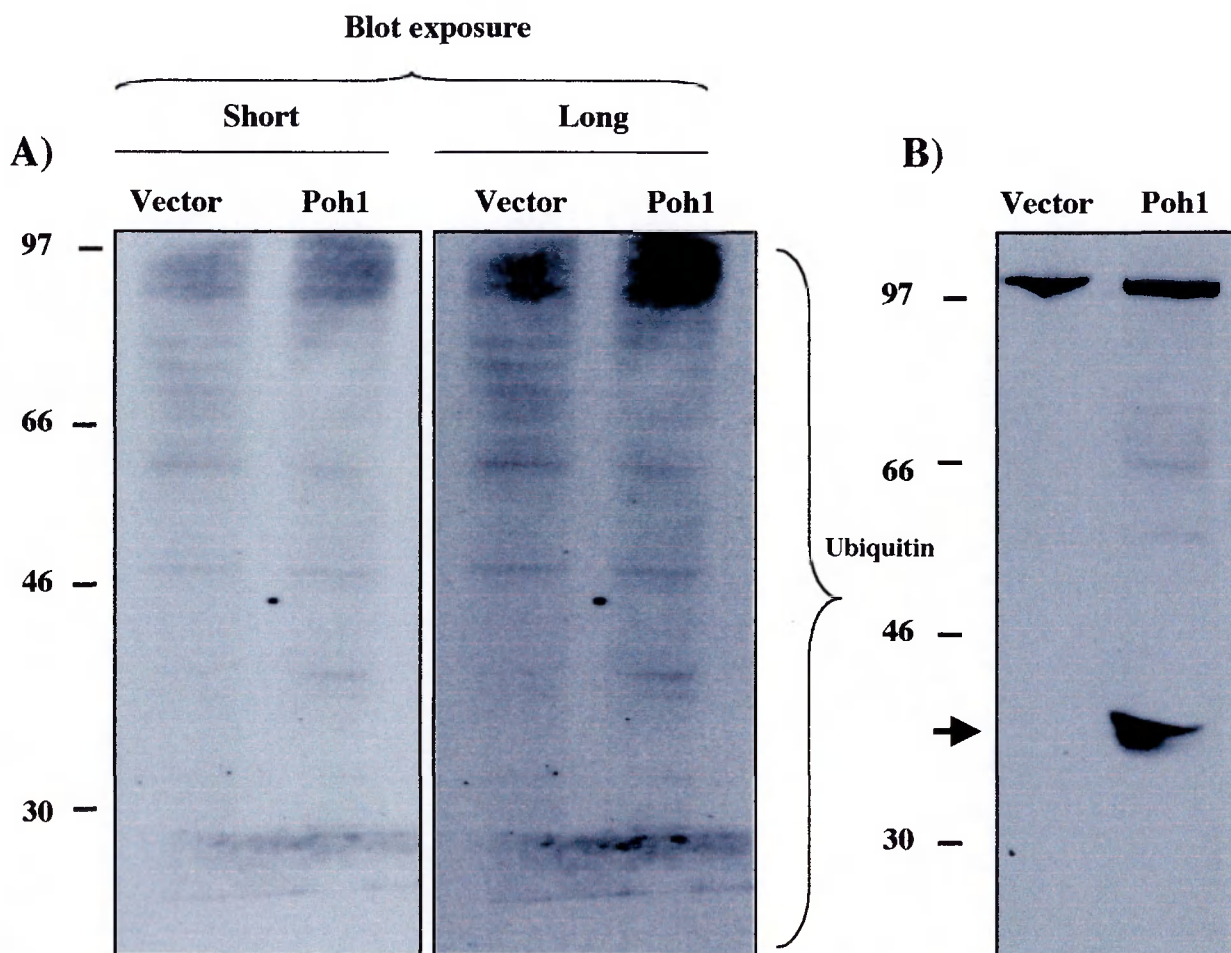


Figure 5.9 - HA-Poh1 overexpression does not affect general protein ubiquitinylation in COS-7 cells

A) Immunoblot analysis of total protein ubiquitinylation in whole cell lysates of COS-7 cells carrying the empty vector or stably expressing HA-Poh1. Protein ubiquitinylation was detected using a rabbit polyclonal anti-ubiquitin antibody. B) Detection of overexpressed HA-Poh1 in the same whole cell lysates as in A using HRP-conjugated monoclonal anti-HA antibody.

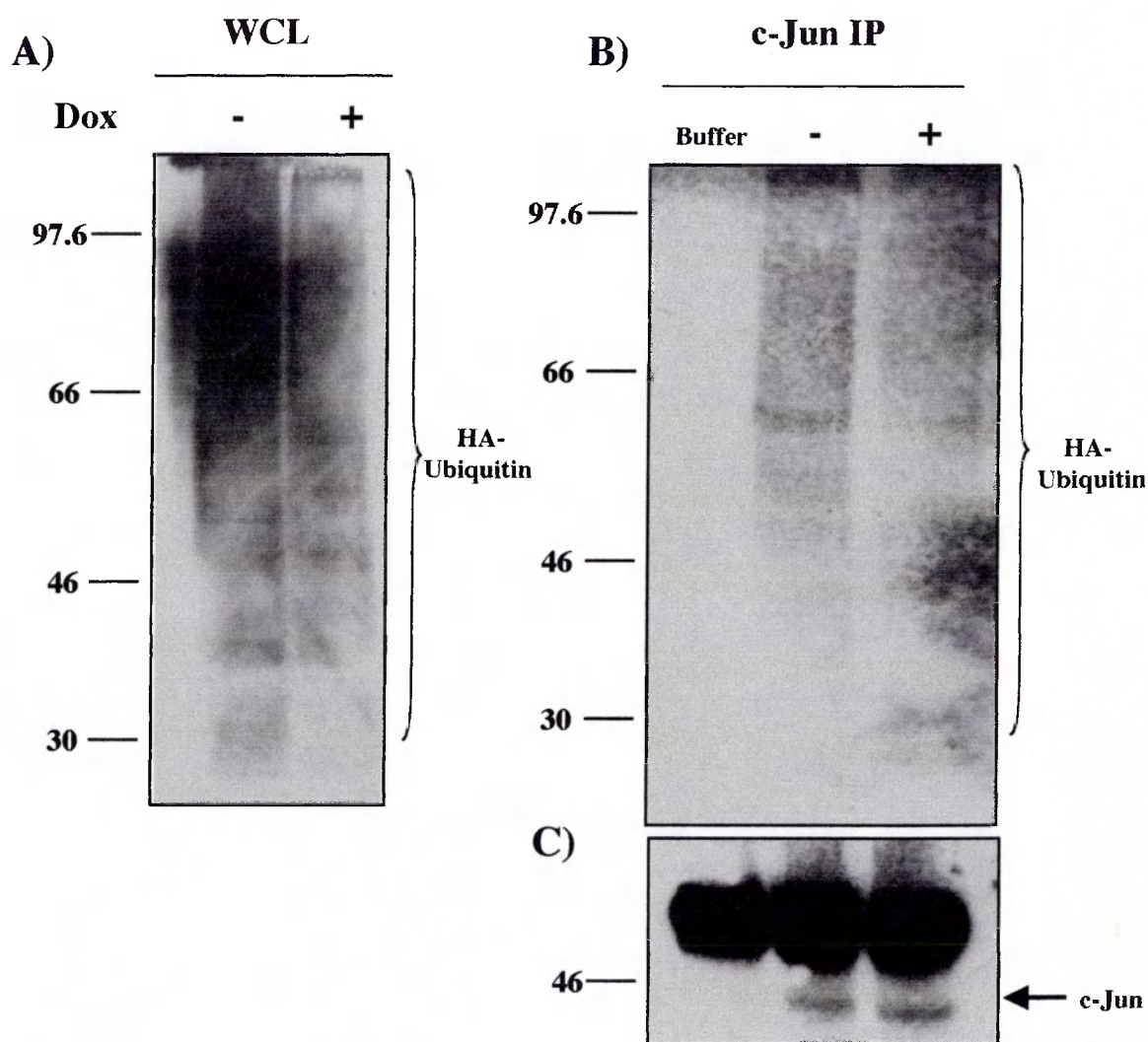


Figure 5.10- Ubiquitinylation level of c-Jun in human cells overexpressing HA-Poh1

c-Jun ubiquitinylation in U2-OS cells from clone 57 mock (-) or doxycyclin (+) treated for 48 hours after transient transfection of pcDNA/HA-Ub vector encoding HA-Ubiquitin. A) Anti-HA immunoblot of whole cell extracts. B) c-Jun detection in α -c-Jun immunoprecipitates. C) Detection of c-Jun ubiquitinylation level in immunoprecipitates using HRP-conjugated monoclonal anti-HA antibody.

ubiquitinated c-Jun bands was observed in doxycyclin-induced cells in comparison with un-induced cells (Figure 5.11B), whereas ubiquitinylation of c-Fos did not show any significant difference between un-induced and induced cells. As a control of transfection efficiency the total level of HA-ubiquitinylation in the different cell lysates before immunoprecipitation was also detected, which was indicative of equivalent transfection efficiency in the different cell populations (Figure 5.11A). The protein level of c-Jun in α -c-Jun immunoprecipitates is also shown, however it was not possible to detect c-Fos in α -c-Fos immunoprecipitates since the protein migrates in close proximity to the IgG band (Figure 5.11C). Detection of c-Jun ubiquitinylation was further repeated but the results were not always reproducible.

Another approach for the study of c-Jun ubiquitinylation was next used. Human U2-OS cells were mock or doxycyclin treated for 24 hours and then transiently co-transfected (1:1 ratio) with pcDNA/HA-Ub and pMT35/c-Jun-His₆, which encodes human c-Jun fused to a His₆-tag sequence at its COOH-terminus. c-Jun-His₆ was purified from extracts of mock or doxycyclin treated cells by nickel chelate affinity chromatography. Lysis buffer alone was used as negative control for protein purification. Although c-Jun protein level, as detected by immunoblotting using a rabbit polyclonal anti-c-Jun antibody, seemed to differ between doxycyclin untreated and treated cells (Figure 5.12A), its ubiquitinylation level, detected using HRP-conjugated monoclonal anti-HA antibody, did not appear to be different (Figure 5.12B).

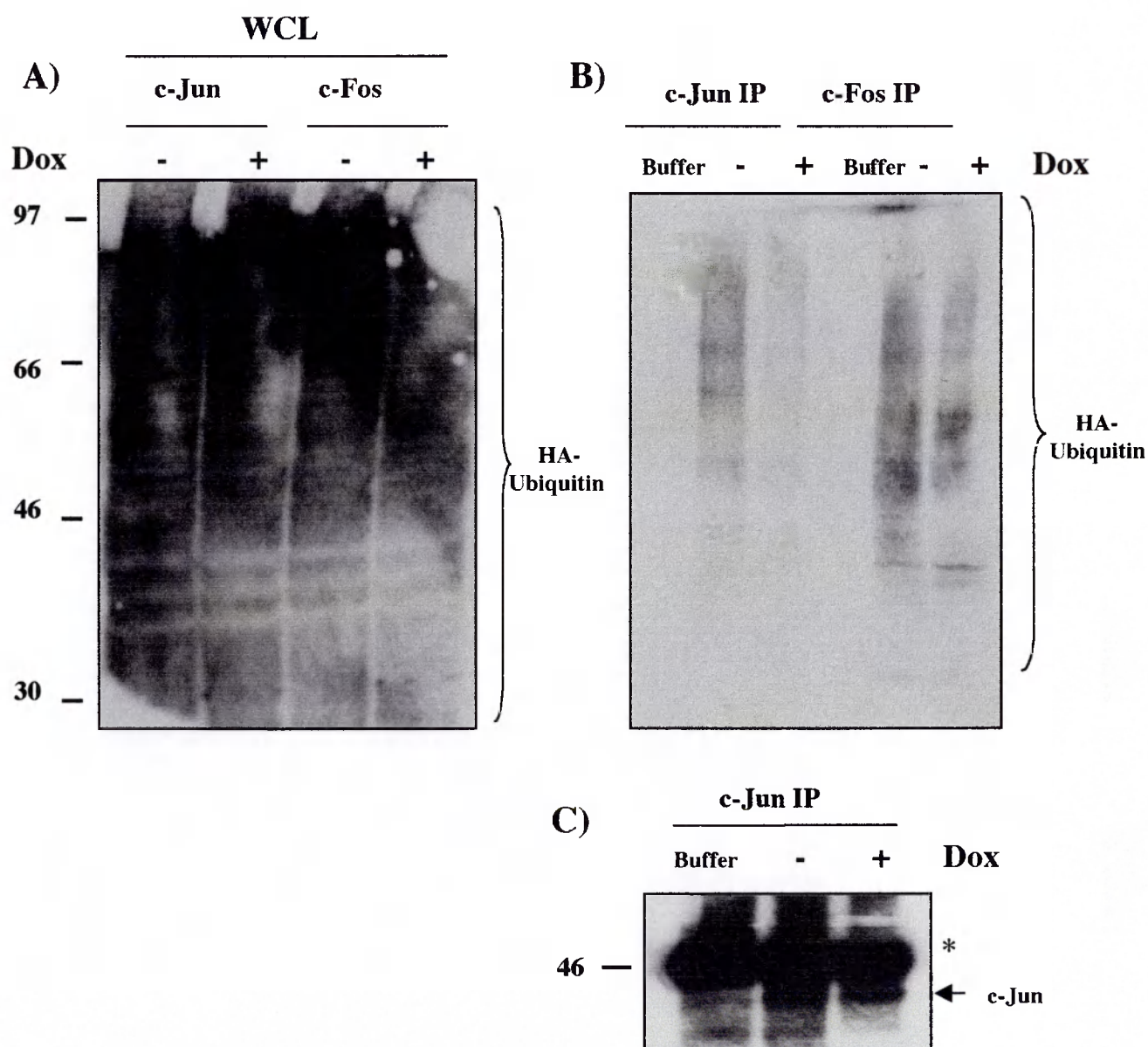


Figure 5.11 - Ubiquitylation level of c-Jun and c-Fos in U2-OS cells overexpressing HA-Poh1

Immunoprecipitation of c-Jun and c-Fos and detection of their ubiquitylation levels in U2-OS cells from clone 57 mock (-) or doxycyclin (+) treated after transient transfection with pcDNA/HA-Ub. A) Anti-HA immunoblot of whole cell extracts B) Detection of c-Jun and c-Fos ubiquitylation level in α -c-Jun and α -c-Fos immunoprecipitates using HRP-conjugated monoclonal anti-HA antibody. C) c-Jun detection in α -c-Jun immunoprecipitates.

* IgG heavy chain

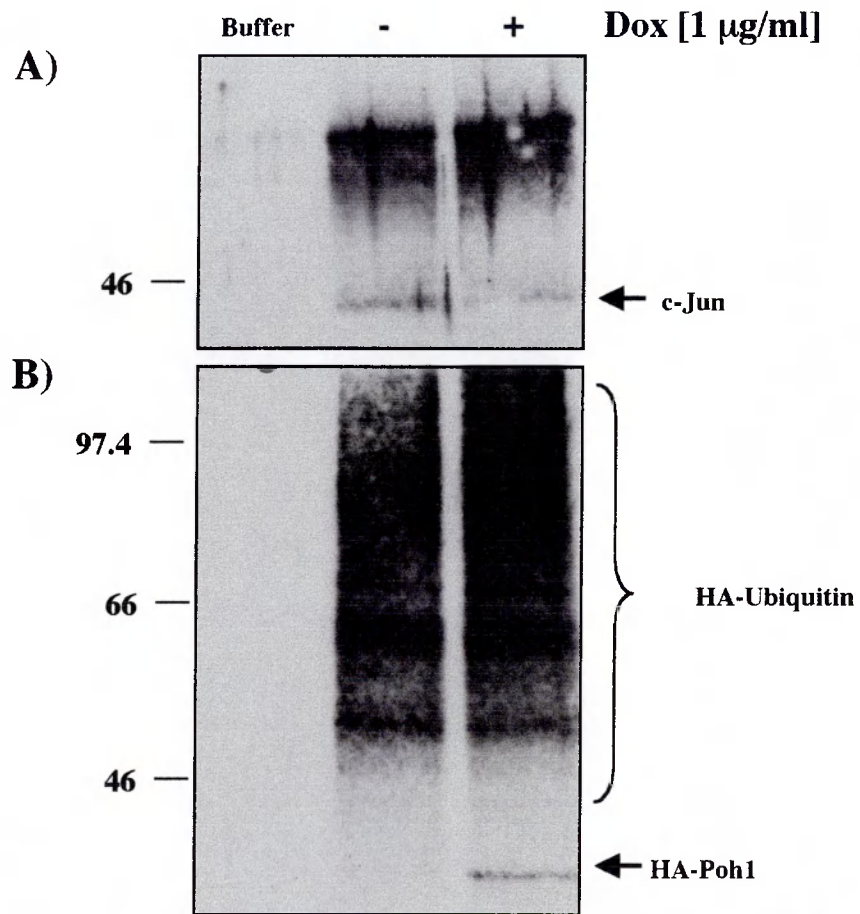


Figure 5.12 – Ubiquitinylation level of c-Jun is not affected by HA-Poh1 overexpression
c-Jun ubiquitinylation level in U2-OS cells from clone 57 mock (-) or doxycyclin (+) treated for 48 hours. A) Purification of c-Jun-His₆ by nickel chelate affinity chromatography after transient co-transfection of cells with c-Jun-His₆ and HA-Ubiquitin expressing vectors. c-Jun was detected by immunoblot analysis using a rabbit polyclonal anti-c-Jun antibody. B) Detection of c-Jun-His₆ ubiquitinylation level after purification by nickel chelate chromatography and immunoblotting using an HRP-conjugated monoclonal anti-HA antibody. Detection of doxycyclin induced HA-Poh1 is also shown.

HA-Poh1 co-immunoprecipitates with c-Jun

By studying the possible effect of overexpressed HA-Poh1 on ubiquitinylation of AP-1 subunits another result came to light. In panel B of Figure 5.12, which shows ubiquitinylation of the c-Jun-His₆ protein purified from human cells, a band of 37 kDa corresponding to overexpressed HA-Poh1 was detected in lysates from doxycyclin treated cells. This result was surprising as the purification of c-Jun-His₆ was performed in presence of guanidinium, which is highly denaturing. That might suggest that the His₆-tagged protein purification was not clean and other proteins might also be present in the preparation or alternatively that the two proteins might associate through an interaction not dissociable under denaturing conditions, such as a covalent link. To investigate a possible interaction further, a more conventional approach was chosen. c-Jun was immunoprecipitated from human cells, mock or doxycyclin treated for 24 hours, and then c-Jun and HA-Poh1 were detected using rabbit polyclonal anti-c-Jun and HRP-conjugated monoclonal anti-HA antibodies, respectively (Figure 5.13A and B). A band corresponding to HA-Poh1 was detected in c-Jun immunoprecipitates from doxycyclin-treated cells (panel B, lane (+)), indicative of an association between the two proteins, whereas endogenous Poh1 was not able to bind to c-Jun, as detection of Poh1 using rabbit polyclonal anti-Poh1 CN13 antibody (panel C) did not show any specific signal. These results are in support of the previous observation (Figure 5.12) and further demonstrate that exogenous but not endogenous Poh1 is able to associate with c-Jun. Whether or not the two proteins interact directly remains to be established.

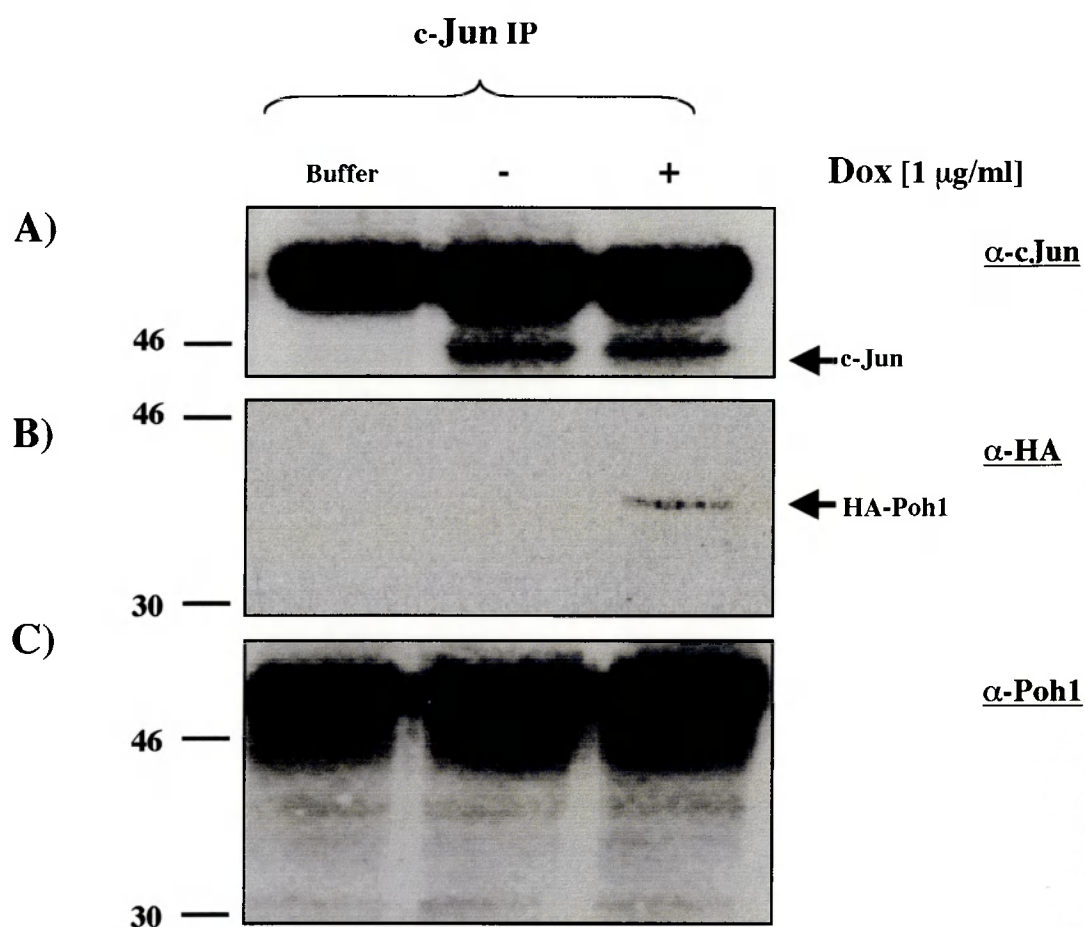


Figure 5.13– Exogenous HA-Poh1 co-immunoprecipitates with c-Jun in human extracts

Immunoprecipitation of c-Jun from U2-OS clone 33 cells, mock (-) or doxycyclin (+) treated for 48 hours. Immune complexes were subjected to immunoblot analysis and detection of c-Jun (A), HA-Poh1 (B) or Poh1 (C) using rabbit polyclonal anti-c-Jun or HRP-conjugated monoclonal anti-HA, rabbit polyclonal anti-Poh1 CN13 antibodies, respectively. Lysis buffer alone was used as negative control.

HA-Poh1 overexpression increases c-Jun protein stability

To further investigate how HA-Poh1 overexpression might cause up-regulation of c-Jun protein level and consequently increase AP-1 activity, c-Jun protein stability was next studied. To this end, U2-OS cells were mock or doxycyclin treated for 48 hours and cell extracts were prepared in order to perform an *in vitro* degradation assay.

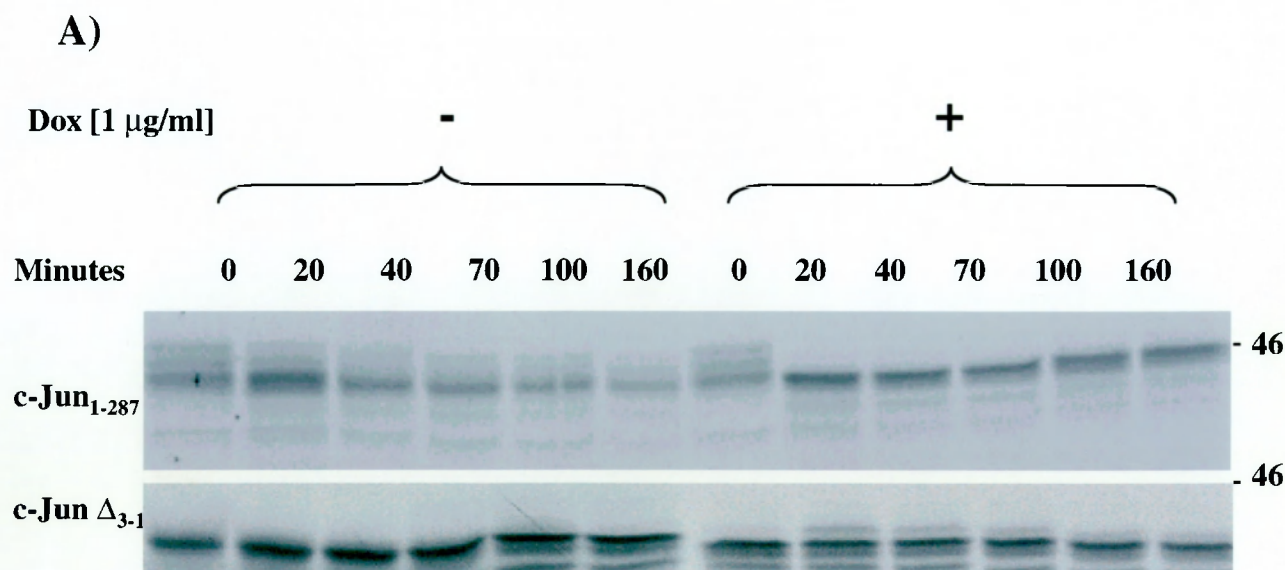
Two different substrates (both a generous gift from Dr. D. Kardassis) were used.

- pcDNA1/c-Jun₁₋₂₈₇ encodes human c-Jun lacking the leucine zipper domain; the protein can still be ubiquitinated and degraded by the 26S proteasome pathway
- pcDNA1/c-Jun Δ_{3-122} expresses human c-Jun lacking the δ -domain, which contains the site of ubiquitination; the substrate is used as a negative control since the protein cannot be ubiquitinated or degraded by the proteasome

Both plasmids were used to generate [³⁵S]-labelled *in vitro* translated protein using the TNT quick transcription/translation system which exploits T7 promoter of the pcDNA1 vector.

Equal amounts of each extract were incubated with an energy-regenerating system and ATP before adding the [³⁵S]-labelled *in vitro* translated c-Jun₁₋₂₈₇ or c-Jun Δ_{3-122} . *In vivo* c-Jun is a short-lived protein with a half-life of 90 minutes (Treier et al. 1994), therefore time points of 0, 20, 40, 70, 100 and 160 minutes were chosen. The reactions were performed at 30°C and at each time point were stopped through the addition of an equal amount of 2x SDS sample buffer. Samples were subsequently separated by 12% SDS-PAGE. The substrate c-Jun₁₋₂₈₇ was degraded in cell extracts from mock treated cells showing a half-life of approximately 90 minutes, in accord with the one previously reported (Treier et al. 1994). In contrast, extracts from cells

treated with doxycyclin showed a slower degradation rate of c-Jun₁₋₂₈₇, indicative of an elevated stability of the protein in cells overexpressing HA-Poh1 (Figure 5.14A). As expected, the protein level of the negative control substrate c-Jun Δ_{3-122} did not change over the same time course. Densitometric analysis of c-Jun₁₋₂₈₇ protein level is also presented and expressed as the mean of the results observed in two independent experiments (Figure 5.14B). It is worthy to note the disappearance of the three upper bands in respect to c-Jun₁₋₂₈₇ corresponding to the phosphorylated forms of c-Jun, which was observed already after 20 minutes during the time-course in the doxycyclin-treated cells only.



B)

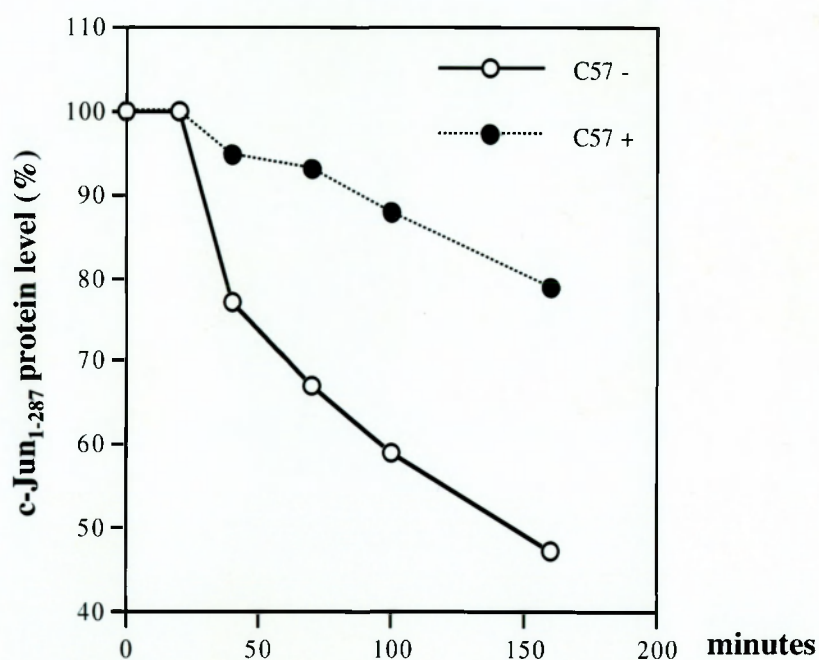


Figure 5.14 - HA-Poh1 overexpression increases c-Jun protein stability

A) *In vitro* degradation assay of human c-Jun₁₋₂₈₇, lacking the leucine zipper domain, and human c-Jun Δ₃₋₁₂₂, deleted in the δ domain, as a negative control, after ³⁵S-labelled *in vitro* protein translation using TNT T7 quick transcription/translation system. Samples were collected at time points 0, 20, 40, 70, 100 and 160 minutes. B) Densitometric analysis of c-Jun₁₋₂₈₇ protein level obtained in two independent experiments. Results are expressed as a percentage of degraded substrate in respect to the amount of protein at time 0.

Discussion

It is shown here that constitutive or inducible overexpression of the highly conserved 26S proteasome regulatory subunit Poh1 in monkey COS-7 and human U2-OS cells, respectively, led to elevated AP-1 transcriptional and DNA binding activities and in addition to an up-regulation of c-Jun and c-Fos protein levels.

In line with these results, the overexpression of *S.pombe* Pad1 is able to elevate the transcriptional activity of Pap1 (Shimanuki et al. 1995), and pleiotropic resistance conferred by exogenously expressed HA-Poh1/Pad1 and other determinants of drug resistance in *S.pombe* is genetically dependent on *pap1*⁺ (see Chapter 1.2) (Claret et al. 1996; Crane et al. 2000; Shimanuki et al. 1995; Spataro et al. 1997; Toda et al. 1992). In contrast, modulation neither of Pap1 protein level nor of its DNA binding activity after exogenous expression of Pad1 or other determinants of drug resistance in *S.pombe* was reported (Crane et al. 2000; Shimanuki et al. 1995; Toda et al. 1992). In addition, a recent report described a role played by the budding yeast Poh1 homologue Rpn11 in the activation of the AP-1-like transcription factor Gcn4 following exposure to UV light (Stitzel et al. 2001).

This body of evidence highlights the key role played by the basic leucine zipper transcription factor Pap1 in the modulation of drug resistance in *S.pombe*. It also suggests that different determinants, if overexpressed or deregulated, are able to activate a common pathway with the consequent transcriptional activation of downstream targets which positively mediate cell survival. The results presented here also provide evidence that at least some aspects of this response to cytotoxic agents are conserved between fission yeast and human cells.

Elevated stability of c-Jun and a possible alteration of its steady state ubiquitinylation level was also observed in HA-Poh1 overexpressing cells. As shown in figure 5.14, the increased stability of c-Jun was coincident with the dephosphorylation of the protein in the doxycyclin-treated cells. This would be in contrast with the JNK-dependent phosphorylation of c-Jun *in vivo* at ⁶³Ser and particularly ⁷³Ser and its subsequent stabilization and transcriptional activation (Fuchs et al. 1997). Nevertheless, the nature of the c-Jun phosphorylated forms in the reticulocyte lysates still awaits clarifications. The 26S proteasome regulatory particle of which Poh1 is a component (Glickman et al. 1998b; Spataro et al. 1997) is necessary for the recognition of ubiquitinated substrates (Deveraux et al. 1994) and the 26S proteasome is the major site of degradation of both c-Jun and c-Fos. This body of evidence together with the modulation of AP-1 by HA-Poh1 presented here sheds light on a possible regulatory network involving proteolysis, transcriptional regulation and drug resistance.

Exogenously expressed HA-Poh1 but not endogenous Poh1 co-immunoprecipitated with c-Jun. In line with this finding, Pad1/Poh1 and Jab1 were reported to share similarity at their NH₂-terminus. Jab1 (CSN5) is a subunit of the COP9 signalosome complex and was initially identified as a protein able to interact with the NH₂-terminus of c-Jun and as a transcriptional co-activator of c-Jun (Claret et al. 1996; Seeger et al. 1998). In addition, Jab1 can confer drug resistance when expressed in *S.pombe* through a Pap1-dependent mechanism and the protein was defined as the first mammalian co-activator able to modulate gene expression in yeast (Claret et al. 1996). The complexes in which Poh1 and Jab1 are associated, the 26S proteasome lid and COP9 signalosome, share common structural features (Aravind, 1998;

Hofmann, 1998) and they have been proposed to have a common evolutionary origin (Wei and Deng 1999).

Bearing in mind all these data, it is reasonable to propose a general model in which elevated levels of HA-Poh1 confer pleiotropic drug resistance through modulation of AP-1 activity in mammalian cells. HA-Poh1 may increase the protein stability of c-Jun, and possibly c-Fos, through either direct or indirect interaction with c-Jun resulting in up-regulation of AP-1 activity. This in turn would lead to elevated expression of downstream target genes possibly involved in positive modulation of cell survival or down-regulation of cell death. In line with this hypothesis, AP-1 activity has been reported to be elevated in some multi-drug resistance cancer cell lines (Moffat et al. 1994; Daschner et al. 1999; Ritke et al., 1994) and in some cases has been associated with an up-regulation of the glutathione-S-transferases, whose role in drug detoxification is well established (McLellan and Wolf 1999).

Although these results suggest a molecular mechanism for the drug resistance mediated by HA-Poh1, they do not establish whether or not this resistance is mediated by the 26S proteasome. As shown in Chapter 3, just a minimal part of the exogenously expressed HA-Poh1 seemed to be associated with the 26S proteasome in COS-7 cells. As shown by sucrose gradient the remaining exogenous protein appeared to be part of smaller sized complexes, although no data on the nature of these complexes are yet available.

Two alternative, but not mutually exclusive models are proposed (Figure 5.12).

Model 1 involves a proteasome-independent pathway in which HA-tagged Poh1 that is not associated with the 26S proteasome is able to bind c-Jun, either directly or indirectly, with subsequent inhibition of c-Jun turnover.

Model 2 involves a proteasome-dependent mechanism. A small amount of overexpressed HA-Poh1 can associate with the 26S proteasome lid, by competing with endogenous Poh1 or in addition to it. This in turn may result in the disruption of 26S proteasome function, possibly by shifting the general equilibrium existing among the subunits of the lid, which is no longer able to recognize specific ubiquitinated substrates, such as c-Jun.

Model 1 is preferred for the following reasons. Overexpressed HA-tagged Poh1 in COS-7 cells is mainly found in comparatively small complexes not associated with the 26S complex, whereas all endogenous Poh1 is associated with the 26S proteasome complex. At the same time, exogenous but not endogenous Poh1 can co-immunoprecipitate with c-Jun suggesting an interaction between the two proteins. This interaction might also lead to the inhibition of c-Jun recognition by the E3-ligase and the subsequent reduction of c-Jun ubiquitination, as observed in figures 5.10 and 5.11. Nevertheless, the modulation of c-Jun ubiquitination needs still to be fully demonstrated.

According to previous reports, approximately 0.1-1.0% of c-Jun is ubiquitinated at any one time *in vivo* in line with other proteins that are degraded by ubiquitin-mediated proteolysis (see Treier et al. 1994). The low abundance of multiubiquitinated substrates reflects their rapid turnover with a half-life of approximately 1 minute. With these data in mind, if the HA-Poh1-induced modulation of c-Jun occurred at level of ubiquitination, it is possible that the resulting change in steady state ubiquitinated c-Jun could not be easily detectable. Furthermore, the equilibrium between substrate ubiquitination and deubiquitination, through the activities of isopeptidases or ubiquitin C-terminal hydrolases (reviewed by Ciechanover 1994), might be shifted towards the

deubiquitinylation process. The final result would be no steady state accumulation of ubiquitinated c-Jun. In these models only ubiquitin-dependent degradation of c-Jun is considered, whereas *in vitro* evidence of c-Jun ubiquitin-independent degradation has also been reported (Jariel-Encontre et al. 1995). Modulation of c-Jun stability by HA-Poh1 through an ubiquitin-independent pathway could occur in either case.

Alternatively, a combination of models 1 and 2 could be proposed. HA-Poh1 not associated with the 26S complex may disrupt the 26S proteasome activity, presumably interfering with the recognition of ubiquitinated c-Jun and the consequent deubiquitinylation through the activity of the hydrolases. The amino acid sequence of Poh1 and its fission yeast homologue Pad1 have a putative ubiquitin hydrolase motif, in which the catalytic residue is cysteine 120 (Figure 3.1); it was in fact previously proposed that Poh1 could function as a ubiquitin hydrolase (Voges et al. 1999). However, preliminary experiments in which the putatively catalytic cysteine residue of Poh1 was mutated to alanine (TGT to GCC) showed that Poh1(C120A) is still able to confer drug resistance and to functionally complement a *pad1*-deletion mutant in *S.pombe*, suggesting that the cysteine residue is not required for essential and drug resistance functions in *S.pombe* (Crane 2000).

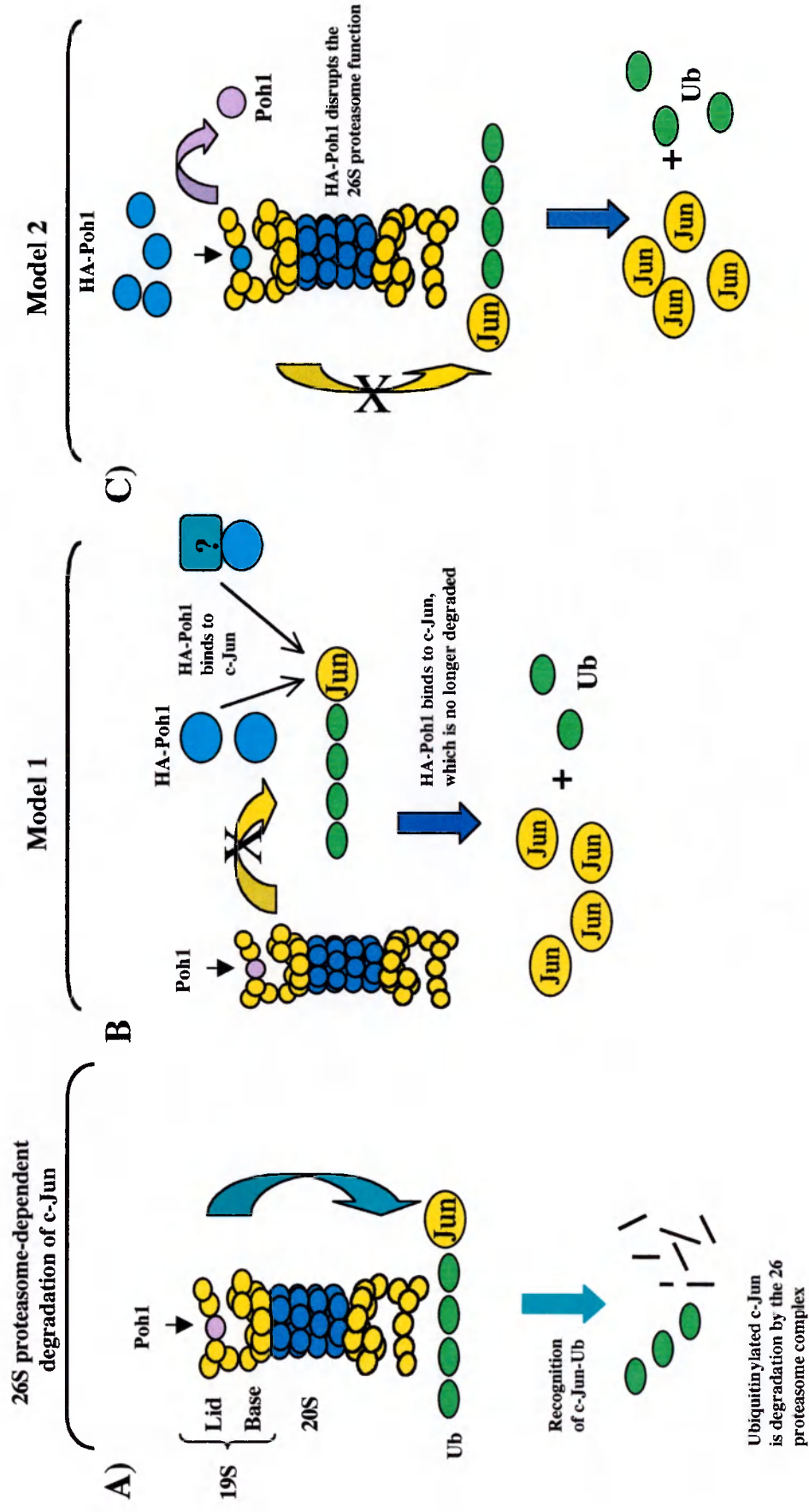


Figure 5.15 - Two models proposed for AP-1 regulation by HA-Poh1 in mammalian cells

- A) The 26S proteasome-dependent proteolysis of c-Jun.
 B) **Model 1** - HA-Poh1 affects c-Jun stability and activity in a proteasome-independent manner.
 C) **Model 2** - c-Jun stability is increased by a 26S proteasome-dependent mechanism.

Chapter 6

The human WD-repeat Pwp1 protein and drug sensitivity

Overview

In chapters 6 and 7 I describe and discuss the characterisation of human Pwp1. This study was initiated following the identification of the *S.pombe* *pwp1*⁺ as multi-drug resistance gene (Crane 2000). This study investigated whether the related human *PWPI* might be involved in multi-drug resistance in mammalian cells.

In this chapter I mainly introduce the WD-repeat protein family and describe what is known about one member of this family Pwp1 in different species (*S.pombe*, *S.cerevisiae* and human). I show that the fission yeast and the human genes are not functional homologues and that overexpression of human *hPWPI* in mammalian cells leads to pleiotropic drug sensitivity rather than drug resistance. The possible mechanism leading to this phenotype is also discussed.

Introduction

The WD-repeat family

The WD-repeat is a large family of regulatory proteins characterised by a divergent unit repeated four to eight times within each polypeptide. Each repeat motif usually ends with the sequence Trp-Asp (WD). Proteins of this family are found in all eukaryotes but not in the prokaryotes. The WD-repeat family is also called the β -transducin family since this motif was first identified in the β -subunit of the heterotrimeric GTP-binding proteins (G proteins), which are involved in signal transduction (reviewed by Neer et al. 1994; van der Voorn and Ploegh 1992).

The repeated unit consists of a region of variable length followed by a core of more or less constant length bracketed by two characteristic pattern elements, GH (Gly-His) and WD.

$$\{X_{6-94} \text{ --- } [GH \text{ --- } X_{23-41} \text{ --- } WD]\}$$

Most repeats are 36-46 amino acids long from WD to WD suggesting that this may represent the primordial length within which insertions or deletions have subsequently taken place. The WD repeat unit can be divided in the relatively conserved elements A and B which are separated by regions that are variable in sequence and length. The most important features of the repeat are the LxGH sequence in element A and the [DN]xxxxx[W,F,Y] [D,N] pattern in element B. The connecting regions n1 and n2 contain many charged residues and prolines. It seems that element A is dispensable since it is absent from some members of the family such as Pwp1 and Cdc20. In these cases no sequence conservation between regions upstream of part B has been observed (Neer et al. 1994).

What is the function of the WD-repeat motif in the proteins? Its presence implies some common structural feature that may or not involve a functional relationship. In fact, the members of the WD-repeat family do not share any functional relationship and the cellular localization of these proteins is also different (reviewed by Neer et al. 1994; van der Voorn and Ploegh 1992). In addition, most of the members of the WD-repeat family are regulatory proteins (Neer et al. 1994). Given that tryptophan (Trp) is the least abundant and least mutable amino acid present in nature, it occupies a unique place in the protein structure and evolution. The presence of tryptophan in the WD-repeat motif would point towards a particular role in the function of these proteins and it has been suggested that the Trp residue could be involved in "stacking" interactions (Duronio et al. 1992).

WD-repeat proteins can also interact with other proteins through the WD-repeat motif with the subsequent formation of multiprotein complexes. For example, G β -subunit in the heterotrimeric GTP-binding proteins associates tightly with G γ -subunit and simultaneously interacts with more than 15 different proteins (reviewed by Smith et al. 1999)). It has also been observed in yeast that some WD-repeat proteins genetically interact with members of the tetratricopeptide repeat (TPR) protein family. TPR-proteins contain multiple repeats 34 amino acids long of unknown function (van der Voorn and Ploegh 1992). As for the WD-repeat proteins, members of the TPR family are involved in many cellular regulatory processes and show different sub-cellular localizations. It has been suggested that the WD and the TPR motifs could form the basis of protein-protein interactions (van der Voorn and Ploegh 1992). Although both families exist in humans, it is still not clear whether members of the WD and the TPR families can interact in human and the possible nature of this interaction.

The G β subunit is the only WD-repeat protein whose crystal structure is known (Garcia-Higuera et al. 1998; Sondek et al. 1996). The seven WD repeats are arranged in a ring in order to form a propeller structure with seven blades. Each blade of the propeller consists of a four-stranded anti-parallel β sheet oriented in such a way that the outer surfaces of the torus are composed of the sheet edge, whereas the turns protrude from the two flat surfaces. It is likely that all the WD-repeat proteins form propeller structures, even if the WD-repeat motif is not essential to form a propeller. In fact, other families of proteins with no homology to the WD-repeat proteins form propellers whose blades are very similar to those in the G β subunit (reviewed by Garcia-Higuera et al. 1998).

PWP1 in different species

S. cerevisiae PWP1

S. cerevisiae PWP1 (ScPWP1) (*p*eriodic *t*ryptophan *p*rotein 1) was first identified in 1992 (Duronio et al. 1992) as a gene divergently transcribed from the Myristol-CoA:protein N-myristoyltransferase (*NMT1*) locus. The *NMT1* gene encodes an enzyme that catalyses the co-translocation attachment of the myristate via an amide bond to the Gly₂ residue at the NH₂-terminal of some eukaryotic and viral proteins. These proteins are usually involved in the regulation of cell growth and differentiation. The translational start of *PWP1* and *NMT1* is 664 nucleotides distant and the genes are transcribed in the opposite orientation (Duronio et al. 1992).

The ScPwp1 amino acid sequence contains two acidic domains located in the amino-terminal and the carboxy-terminal of the protein (residues 23-109 and 524-276). Furthermore, the ScPwp1 amino acid sequence has eight variable-length internal repeats, each containing an invariant tryptophan residue, a feature of proteins

belonging to the WD-repeat protein family (Duronio et al. 1992). A positive charged cluster (residues 268-287) containing a putative nuclear localization signal (NLS) Lys-Lys-Lys-Ser-Lys (residues 273-277) was also identified. It has been proposed that basic highly charged region may be a feature of the regulatory proteins, such as the transcription factors (Brendel and Karlin 1989).

Sc*PWPI* is a non-essential gene, but the deletion mutants grow slowly compared to wild type budding yeast (Duronio et al. 1992). The deletion mutants show also a reduced global protein synthesis.

ScPwp1 shares similarity at the amino acid sequence level with Prp4 (pre-mRNA processing) (Duronio et al. 1992). Prp4, encoded by the essential gene *PRP4*, is a member of the WD-repeat family with predicted serine/threonine kinase activity and is the first putative protein kinase found to be involved in the process of mRNA splicing (Alahari et al. 1993; Gross et al. 1997). Prp4 is a component of the U4/U6 snRNP particle, which mediates the splicing of nuclear pre-mRNA. The structural similarity between the Prp4 and the Pwp1 sequences suggests that the two proteins may belong to the same subgroup of the WD-repeat family with a putative nucleotide binding activity (Duronio et al. 1992). It was speculated that the aromatic Trp residues might function similarly to the aromatic phenylalanine (Phe) residue, which is found in RNA-binding proteins containing the ribonucleoprotein consensus sequence and interacts directly with nucleotides (Duronio et al. 1992).

Human *PWPI*

Human *PWPI* (h*PWPI*) was first described in 1994 (Honore et al. 1994). The protein has an open reading frame (ORF) of about 1.6 kb and a molecular mass of 79.4 kDa. It contains 9 Trp residues some of which are localised in conserved

repeats and a putative nuclear localization signal (NLS) (KKKGK). The 2.4 kb mRNA is ubiquitously expressed with high amount in placenta, liver, skeletal muscle, kidney and pancreas. It was shown by cell fractionation that hPwp1 protein was more abundant in the nucleus than in the cytoplasm of the human cell (Honore et al. 1994). The protein sequence also contains some putative phosphorylation sites and by cell [³²P]-phosphate labelling and bidimensional (2-D gel) electrophoresis it was observed that the protein was, in fact, phosphorylated (Honore et al. 1994).

S. pombe pwp1⁺

S.pombe pwp1⁺ (*Sppwp1⁺*) was recently identified as a novel multi-drug resistance determinant during a fission yeast cDNA library screen (Crane 2000). Overexpression of fission yeast *pwp1⁺* under the control of the *nmt1* thiamine-repressible promoter from pREP3X vector (Maundrell 1993) caused resistance to MBC, caffeine, cadmium and staurosporine. The multi-drug resistance phenotype conferred by SpPwp1 was dependent on fission yeast Pap1 (Crane 2000), previously observed for other determinants of multi-drug resistance in fission yeast (see Introduction Chapter 1).

Overexpressed HA-tagged SpPwp1 in fission yeast localised in the cytoplasm and after sucrose density gradient centrifugation the protein was detected in fractions containing very large sized complexes.

S.cerevisiae PWP1-deleted cells grew slowly in comparison with the wild-type (Duronio et al. 1992). In contrast, fission yeast *pwp1⁺* is essential, as the disruption of its open reading frame (ORF) caused lost of cell viability (Crane 2000).

Results

Human HA-PWPI in fission yeast

S.pombe pwp1⁺ was recently described as multi-drug resistance gene (Crane 2000). Fission yeast *pwp1*⁺ and human *PWPI* share 34% of identity at the amino acid level (Figure 6.1), so the possibility that this phenotype was conserved from yeast to human was explored.

To investigate if human *PWPI* is a functional homologue of *S.pombe pwp1*⁺, the human gene was first expressed in fission yeast cells. The full-length human cDNA sequence, a generous gift of Dr. J.Celis (Arhaus University, Denmark), was amplified using polymerase chain reaction (PCR) and cloned in the fission yeast expression vector pREP3X (by S.Carobbio), which expresses the gene under the control of the *nmr1* thiamine-repressible promoter (Maundrell 1993). The pREP3X vector was previously modified by insertion of a triple HA-epitope tag sequence in its multicloning site (by R.Craig) and the 5'-end of hPWP1 sequence was fused to the 3'-end of the HA sequence. The vector pREP3X/HA-hPWP1 was transformed by electroporation into *S.pombe leu1-32* cells and the plasmid-containing cells were selected by plating them onto EMM2 agar plates lacking leucine. Cells were then grown in presence or in absence of thiamine [10 µg/ml] for at least 16 hours in order to allow the expression of the gene and hPwp1 protein was detected by immunoblot analysis using an HRP-conjugated monoclonal anti-HA antibody (Figure 6.2). The exogenous human protein migrated approximately at 80 kDa, in accord with the size of 79.4 kDa of the human protein (Honore et al. 1994). In contrast, when the *nmr1* promoter was repressed through the addition of thiamine no expression of the HA-hPwp1 protein was observed (Figure 6.2).

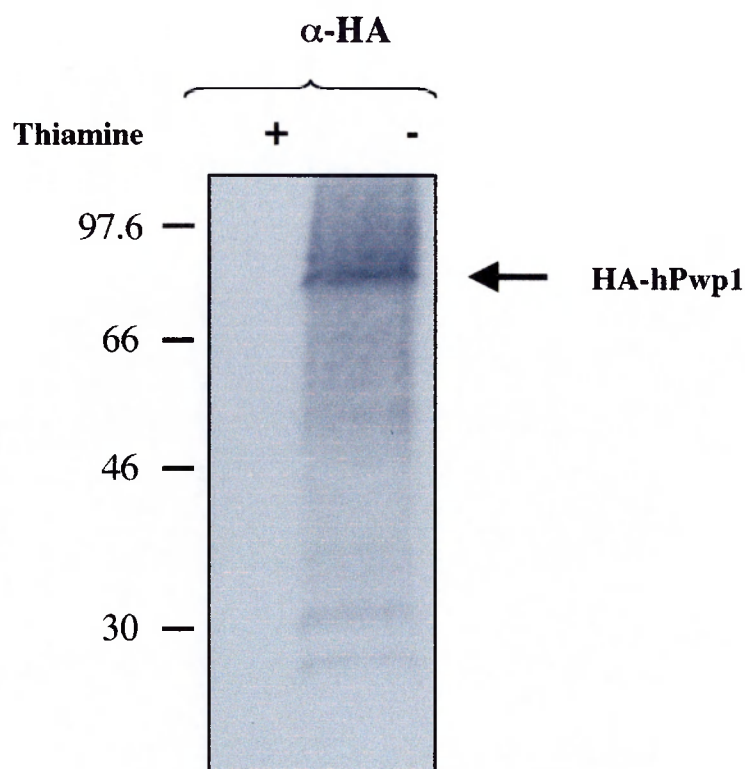


Figure 6.2 – Expression of human HA-hPwp1 in *S.pombe* cells

Immunoblotting of human HA-tagged Pwp1 in fission yeast. Cells carrying the pREP3X/HA-hPWP1 plasmid were grown with (+) or without (-) thiamine for 16 hours. Detection of HA-hPwp1 protein was performed by immunoblot analysis using HRP-conjugated monoclonal anti-HA antibody.

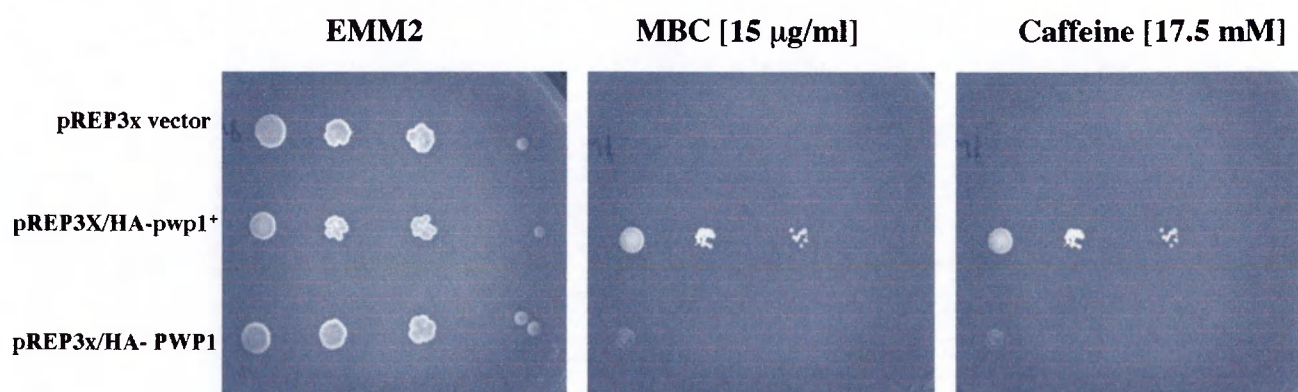


Figure 6.3 – Expression of human Pwp1 in *S.pombe* does not confer pleiotropic drug resistance

S.pombe leu1-32 cells transformed with pREP3X, pREP3X/HA-pwp1⁺ and pREP3X/HA-PWP1 were grown for 16 hours in the absence of thiamine. Serial dilutions of each culture (10000, 1000, 100 and 10 cells) were plated as indicated onto EMM2 agar containing no drug (left panel), 15 µg/ml MBC (middle panel) or 17.5 mM caffeine (right panel). Plates were incubated at 30°C for 3-4 day and photographed.

Next, the capacity of the human protein to confer pleiotropic resistance to cytotoxic drugs in fission yeast was investigated. Fission yeast cells were transformed with the empty vector (pREP3X) or the vector expressing the HA-tagged *S.pombe pwp1*⁺, as a positive control, or human *PWP1*. The transformed cells were plated onto the agar plates containing MBC, caffeine or no drug (Figure 6.3). As previously shown (Crane 2000), overexpressed *S.pombe pwp1*⁺ is able to confer pleiotropic resistance after exposure of the cells to MBC and caffeine, whereas overexpressed *hPWP1* did not induce this phenotype.

S.pombe pwp1⁺ is an essential gene and the expression of the human gene in *S.pombe pwp1*⁺-deleted mutants was not able to rescue the viability of the cells (Crane 2000).

These results taken together suggest that the *S.pombe* and human *pwp1*⁺/*PWP1* genes are not functional homologues.

Stable expression of hPwp1 in mammalian cells

To characterise human *PWP1* in mammalian cells, tetracycline repressible expression (see Introduction of Chapter 4) was first attempted in human cells using pRetro OFF vector. In this "Tet-off" system tetracycline acts as repressor. This system was attractive because a single vector contains all the essential elements for regulatable gene expression (see A.4 - Appendix).

1. The *tTA* gene encoding the tetracycline transactivator which regulates the expression of the gene of interest
2. A multicloning site (MCS) for the cloning of the gene of interest which is expressed under the control of a tetracycline responsive element (TRE) within the promoter

3. A puromycin resistance gene for stable selection of the transfectants

HA-tagged hPWP1 cDNA was cloned into pRetro OFF vector and expressed in the NCI-H460 human small cell lung cancer line, chosen because it is comparatively sensitive to cytotoxic drugs (C.Norbury, personal communication). Unfortunately, none of the puromycin-resistant clones obtained expressed HA-hPwp1 protein, as detected by immunoblot analysis using an antibody against the HA-epitope tag of the protein. Expression of HA-Pwp1 was detected only once after transient transfection of the plasmid in these cells (data not shown). Transient transfection of the plasmid in human HeLa cells was also performed but again no expression was seen. Given that the calf serum present in the medium culture may contain small amounts of tetracycline, the possibility that this tetracycline might inhibit expression of the gene was also addressed. Therefore, tetracycline-free serum was tested but the results were unchanged.

Next, the human gene was expressed using isopropyl- β -D-thiogalactopyranoside (IPTG) inducible expression in the human HT1080 fibrosarcoma cell line. A significant number of positive clones stably expressing HA-hPwp1 after induction with IPTG were obtained. Despite this, the degree of induction by IPTG was only very modest. In accord with this result, it has been reported that IPTG acts as a slow and often inefficient inducer leading to moderate induction of gene expression (Gossen and Bujard 1992).

Subsequently, to obtain a better expression level of the exogenous protein inducible expression was substituted with constitutive expression of HA-hPwp1. The HA-hPWP1 open reading frame was cloned into the pcDNA3.1+ mammalian expression vector (pcDNA3.1/HA-hPWP1) and the empty pcDNA3.1+ vector or pcDNA3.1/HA-hPWP1 were co-transfected with the pOPRV-CD2 plasmid into

monkey COS-7 cells at a ratio of 4:1. 24 hours later the CD2⁺ cells were selected using anti-CD2 magnetic beads (see Figure 3.6 in Chapter 3). Cells containing the empty vector or the HA-hPwp1-expressing vector were then selected for two weeks with G-418, in order to generate stable polyclonal populations, and expression of HA-hPwp1 in whole cell lysates from the two cell populations was then analysed by immunoblotting. A band with a molecular mass of approximately 80 kDa was detected only in the cell lysates from HA-Pwp1 expressing cells using HRP-conjugated monoclonal anti-HA antibody (Figure 6.4A). Several stable clones were subsequently derived from the polyclonal pool (see Materials and Methods - Chapter 2) and the expression of HA-Pwp1 in three of them is also shown (Figure 6.4B).

With the aim of identifying also the endogenous protein, polyclonal antibodies against human Pwp1 were prepared using two different approaches. First, a fusion protein of hPWP1 and glutathione-S-transferase (GST) was generated using the pGEX-4T-3 vector. The GST-hPWP1 expressing plasmid was then transformed into *E.coli* BL21 and expression of the fusion protein was induced through the addition of IPTG for two hours. After cell lysis and separation of the proteins by SDS-PAGE, the induced GST-Pwp1 fusion protein was detected by Coomassie blue staining (Figure 6.5A). Next, bacterial cells were fractionated after induction with IPTG and the overexpressed GST-fusion protein was mainly found in the insoluble fraction (Figure 6.5B). This fraction was further used to purify the GST-hPwp1 protein with the purpose of raising polyclonal antibodies against it. Unfortunately, in two independent preparations the anti-hPwp1 antibodies were not able to specifically recognise the human protein by immunoblot analysis, after expression either in COS-7 or in fission yeast cells. Therefore, a second approach was used. Peptides were designed corresponding to the amino-terminus and the carboxy-terminus of the

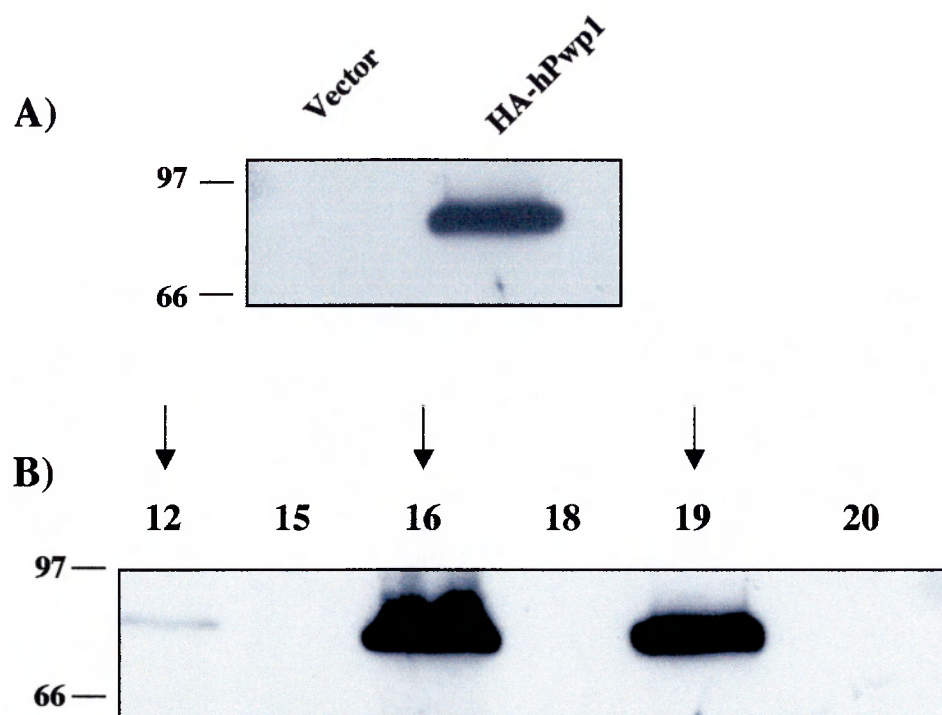


Figure 6.4 – Stable expression of HA-hPwp1 in mammalian cells

Immunoblot analysis of HA-hPwp1 protein. Whole cell lysates were prepared from COS-7 polyclonal pools stably expressing HA-hPwp1 or carrying the empty vector (pcDNA3.1+) (A) and some stable clones derived from the polyclonal population (B). Detection of the protein was carried out using HRP-conjugated monoclonal anti-HA antibody.

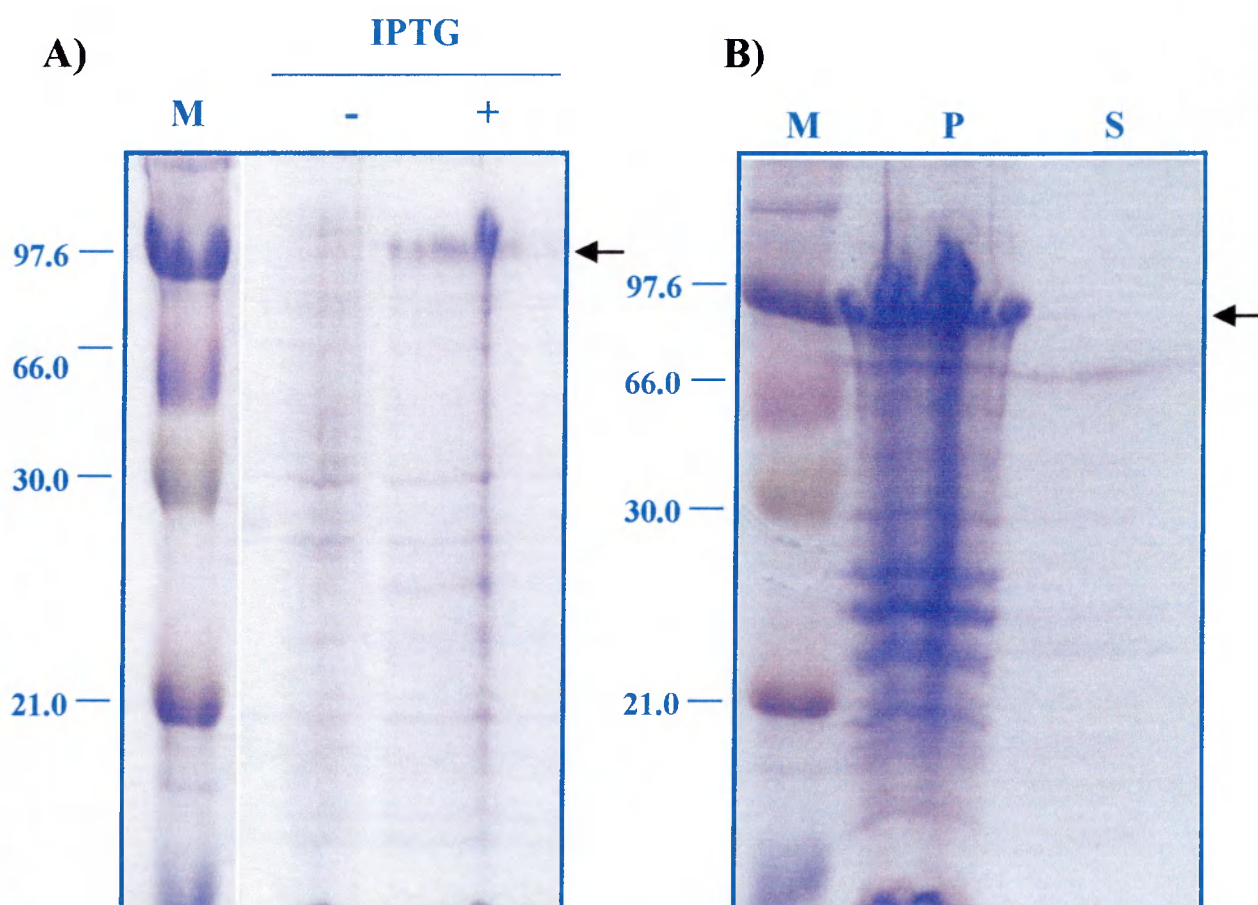


Figure 6.5 - Expression of the GST-hPwp1 recombinant protein in *E.coli*

Coomassie brilliant blue staining of GST-hPwp1 fusion protein expressed in *E.coli* BL21. A) *E.coli* BL21 transformed with pGEX-4T-3/hPWP1 were grown in LB medium to an A_{595} of 0.5. Cells were mock (-) or IPTG [0.5 mM](+) treated for 2 hours in order to induce GST-hPwp1 expression and then lysed in 2x SDS sample buffer. Aliquots from each sample were separated by 10% SDS-PAGE and stained with Coomassie blue. B) After IPTG induction, the bacterial cells were also fractionated by ultracentrifugation and the pellet (P) and the supernatant (S) were collected, lysed in 2x SDS sample buffer and separated by 10% SDS-PAGE. The proteins were stained by Coomassie brilliant blue.

protein. After synthesis (ICRF Biological Resources) the peptides were conjugated with Keyhole Limpet Haemocyanin (KLH), used as carrier, and then used for rabbit immunization. The antibodies were tested by immunoblot analysis and the rabbit polyclonal antibody CN35 was able to recognise the endogenous hPwp1 (Figure 6.6A). A short and a long exposure of immunoblot in panel A of the Figure are shown and in addition to the band of about 80 kDa corresponding to hPwp1 two more slowly migrating bands were identified (lower panel in A). This pattern was always observed using this antibody. Detection of HA-tagged hPwp1 was also carried out in the same cell lysates as a positive control (B).

hPwp1 overexpression confers pleiotropic drug sensitivity

The drug susceptibility of cells stably expressing HA-hPwp1 was investigated using clonogenic survival assays. The survival of COS-7 cells carrying the empty vector or over-expressing HA-hPwp1 was compared after exposure to a given cytotoxic drug for 24 hours. Figure 6.7 shows that overexpression of HA-tagged hPwp1 causes sensitivity to unrelated drugs, such as cisplatin, 7-hydroxystaurosporine (UCN-01) and vincristine sulphate. Given that these drugs have diverse mechanisms of action, I refer to this phenotype as a multi-drug sensitive phenotype.

These results were unexpected. Although the overexpression of the human gene in *S.pombe* did not confer pleiotropic drug resistance (Figure 6.3), it was anticipated that the overexpression of human *PWP1* in mammalian cells might be able to trigger the same cellular response induced by the *S.pombe* gene after exposure of the cells to drugs.

Given that the phenotypes observed in human and fission yeast cells were opposite, it was addressed if the HA-epitope tag fused to the amino-terminus of hPwp1 was

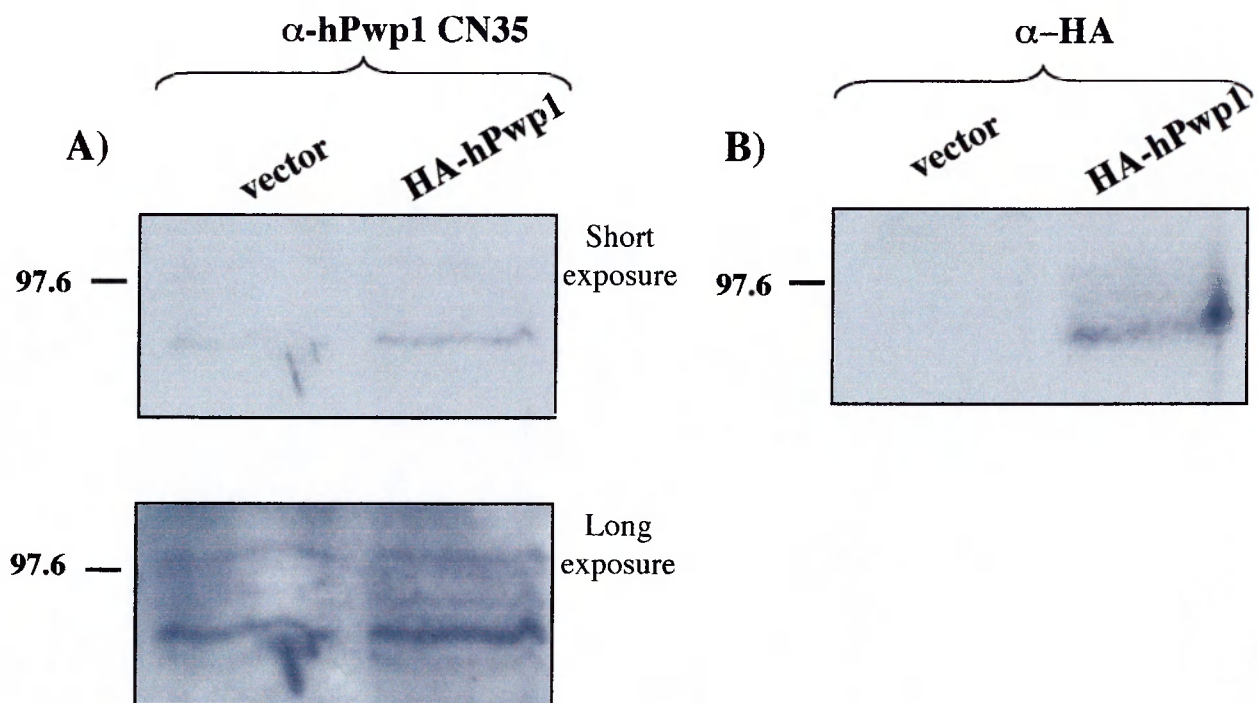


Figure 6.6 – Detection of hPwp1 protein in COS-7 cells using rabbit polyclonal anti-Pwp1 CN35 antibody

Immunoblot analysis of COS7 cell lysates from polyclonal pools stably expressing HA-hPwp1 or carrying the empty vector (pcDNA3.1+). Whole cell lysates were processed by immunoblot using rabbit polyclonal anti-Pwp1CN35 antibody (A) or HRP-conjugated monoclonal anti-HA antibody (B).

somehow disrupting the function of the protein resulting in a dominant-negative phenotype. To this end, the un-tagged *hPWP1* cDNA was cloned into pcDNA3.1+ (pcDNA3.1/hPWP1) and stably expressed in COS-7 cells using the same transfection strategy previously used for the HA-tagged gene. At this stage it was not possible to detect endogenous or un-tagged Pwp1 by immunoblot analysis because the initial preparations of polyclonal anti-Pwp1 antibodies were unsuccessful. On the other hand, as it was observed that HA-tagged *hPWP1* and *POH1* were efficiently expressed from the pcDNA3.1 vector, the drug susceptibility of cells expressing un-tagged hPwp1 was tested. COS-7 cells carrying the empty vector or overexpressing un-tagged hPwp1 were exposed to several concentrations of cisplatin and cell survival or cell viability was measured by clonogenic survival and MTS cytotoxicity assays, respectively (Figure 6.8). Un-tagged hPwp1 induced sensitivity to cisplatin as previously observed for the HA-tagged protein, suggesting that the HA epitope tag is not responsible for hPwp1-conferred sensitivity.

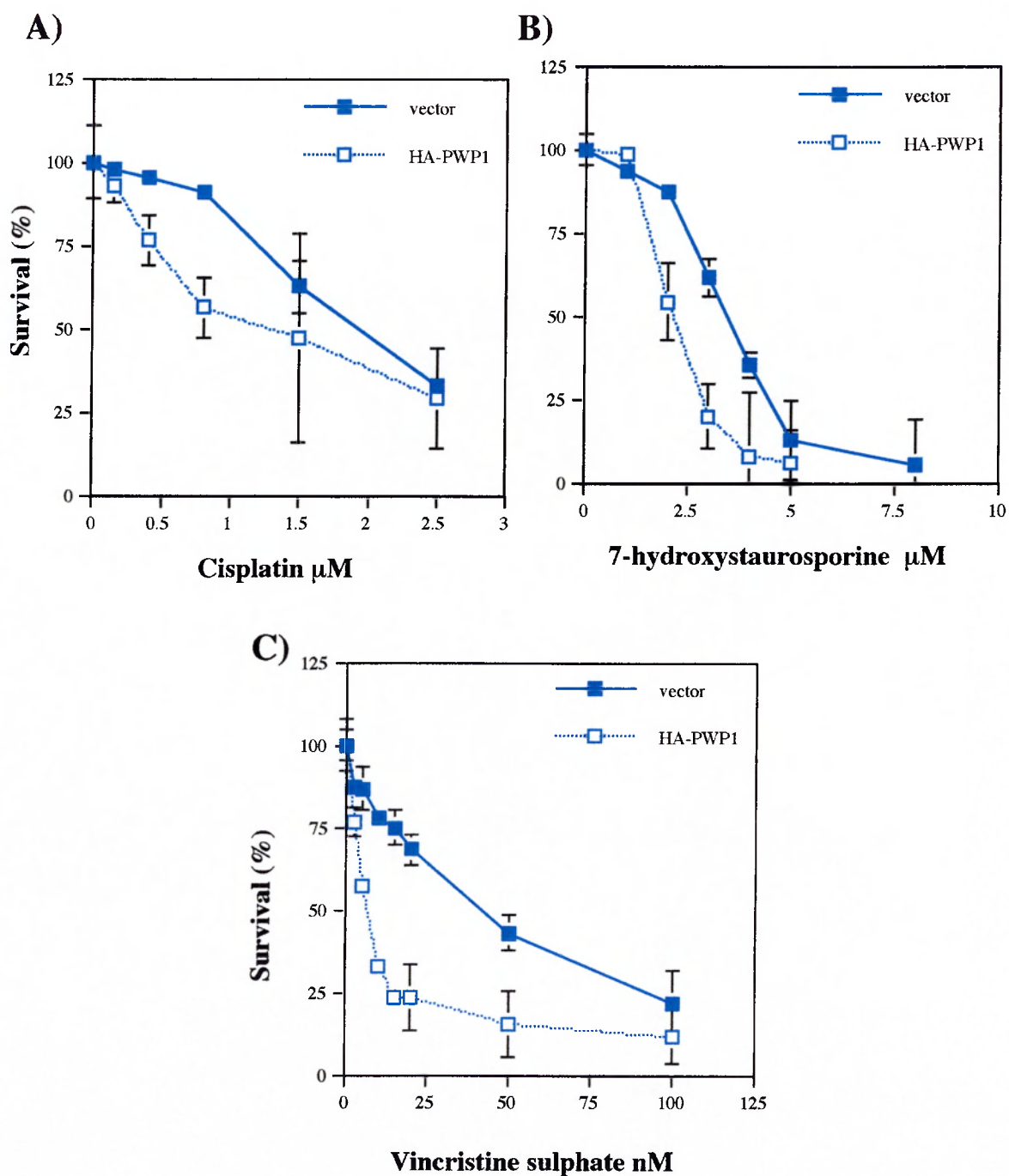


Figure 6.7 - HA-hPwp1 stable expression confers pleiotropic drug sensitivity in mammalian cells

Clonogenic survival curves for COS7 cells stably transfected with the empty vector (pcDNA3.1+) or pcDNA3.1/HA-hPWP1. 3000-4000 cells were plated in triplicate and treated with different concentrations of cisplatin (A), 7-hydroxystaurosporine (B) or vincristine sulphate (C) for 24 hours. Cells were then washed once with PBS and refed with fresh medium. The number of surviving colonies was counted after about 2 weeks and expressed as the percentage of the control (cells with no drug).

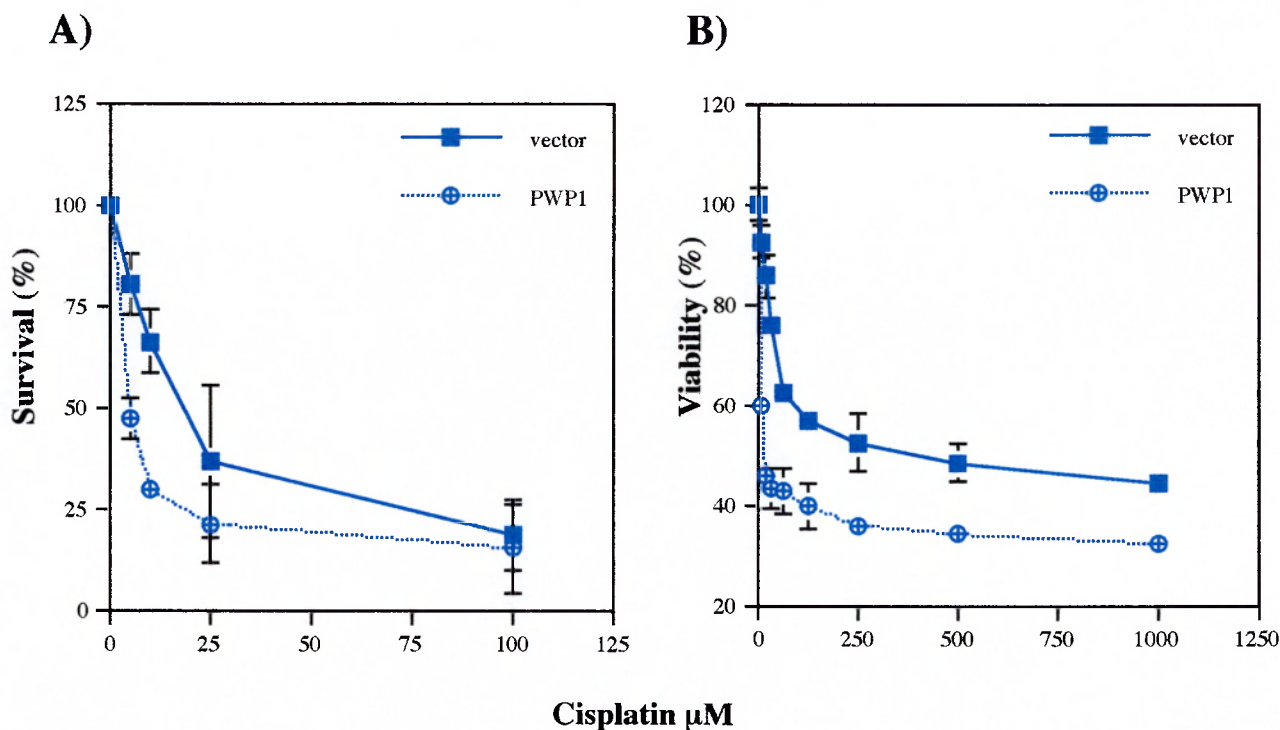


Figure 6.8 - The HA-epitope tag fused to the hPWP1 gene is not responsible for the drug sensitivity phenotype

A) Clonogenic survival curve for COS-7 stably transfected with the empty vector (pcDNA3.1+) or pcDNA3.1+/hPWP1 and exposed to different concentrations of cisplatin for 24 hours. B) MTS cytotoxicity assay for the cells as in A. The number of surviving colonies or viable cells was expressed as the percentage of the survival or the viability of the control (cells with no drug) in A and B, respectively.

Discussion

The study of the human *PWPI* gene in relation to its putative role in multi-drug resistance was undertaken because fission yeast *pwp1*⁺ was recently identified as multi-drug resistance gene (Crane 2000). No data regarding a putative role of the *S.cerevisiae* *PWPI* gene in drug resistance have been described.

Here it is first shown that *S.pombe* *pwp1*⁺ and human *PWPI* are not functional homologues. Expression of the human gene in fission yeast did not show any phenotype and expression of human *PWPI* from a multicopy plasmid could not restore viability to fission yeast *pwp1*⁺-deletion mutants (Crane 2000).

Surprisingly, the presence of exogenously expressed human Pwp1 led to a survival disadvantage of COS-7 cells after exposure to cytotoxic drugs. Although the identity at the amino acid level between the *S.pombe* and the human proteins is just 34% and the two counterparts are not functional homologues, it had seemed possible at the outset of this study that the fission yeast *pwp1*⁺-conferred drug resistance phenotype might be conserved in mammalian cells. The drug sensitivity could not be explained by any deleterious effect of the HA epitope tag fused to the amino-terminus of hPwp1.

How then can the drug sensitivity phenotype be explained?

Two views are possible.

1. A divergent evolution between the fission yeast and human *pwp1*⁺/*PWPI* genes occurred. The signal generated from Pwp1 is interpreted differently in the two species.
2. Human *PWPI* is not the human gene most closely related to *pwp1*⁺ and possibly the true homologous has yet to be found.

Treatment with a variety of cytotoxic drugs has been documented to induce apoptosis in the cells generally by causing cellular or DNA damage. It could be reasonable to assume that human Pwp1 acts as a intermediary of the apoptotic response triggered by cytotoxic stress in mammalian cells. This view could be in accord with no evidence of apoptosis in fission yeast, where *pwp1*⁺ induces the Pap1-dependent stress response that leads to a survival advantage. Apoptosis could be a recent response generated in more complex organisms in order to escape irreparable cellular damage or intolerable environmental stress conditions. Although fission yeast is often used as a potent genetic tool in order to investigate complex processes in eukaryotic cells (Nurse 1990), such as regulation of mitosis and cell cycle, not all pathways or gene functions are conserved.

On the other hand, human *PWP1* could not be the closest counterpart of *S.pombe* *pwp1*⁺.

Distinction between these possibilities will require further information.

The functional role of Pwp1 in the cell is not known. Studies on *S.cerevisiae* have suggested that Pwp1 might have a regulatory role, possibly in cell growth and/or gene transcription (Duronio et al. 1992). The Pwp1 protein has a positive charged domain in the central part of its amino acid sequence, where the putative localization signal KKKGK is also localised (Duronio et al. 1992), which seems to be a feature of many regulatory proteins, such as transcription factors (Brendel and Karlin 1994). This putative regulatory role of Pwp1 is also in line with the evidence that most members of the WD-repeat protein family are regulatory proteins involved in a variety of cellular processes, including cell division, cell fate determination, gene

transcription, transmembrane signaling, mRNA modification and vesicle fusion
(Neer et al. 1994).

Chapter 7

Further characterization of the human PWP1 gene

Overview

In this chapter I describe further studies aimed at a better understanding of the general function of hPwp1 in human cells. I describe the sub-cellular localization of HA-tagged hPwp1 and discuss factors underlying this localization. In addition, I also present preliminary results obtained using anti-sense RNA expression and the two-hybrid screen.

Results

Sub-cellular localization of HA-Pwp1 in COS-7 cells

To better characterize human Pwp1 in mammalian cells several questions were addressed. First, sub-cellular localization of the protein was studied using two different approaches. COS-7 cells stably expressing HA-hPwp1 were grown on sterile glass coverslips in a 6-multi-well plate. The cells were subjected to indirect immunofluorescence using mouse monoclonal anti-HA and FITC-conjugated anti-mouse antibodies (Figure 7.1A). The cells were also stained with FITC-conjugated anti-mouse antibody alone as a negative control (Figure 7.1B). Exogenously expressed HA-hPwp1 was detected by fluorescence microscopy and was present both in nuclear and cytoplasmic compartments of the cells, with a stronger signal in the cytoplasm.

Next, the localization of the protein was studied using a method independent of antibody detection. To this end a fusion protein of hPWP1 and enhanced green fluorescent protein (EGFP) sequences was generated using the pEGFP vector. The

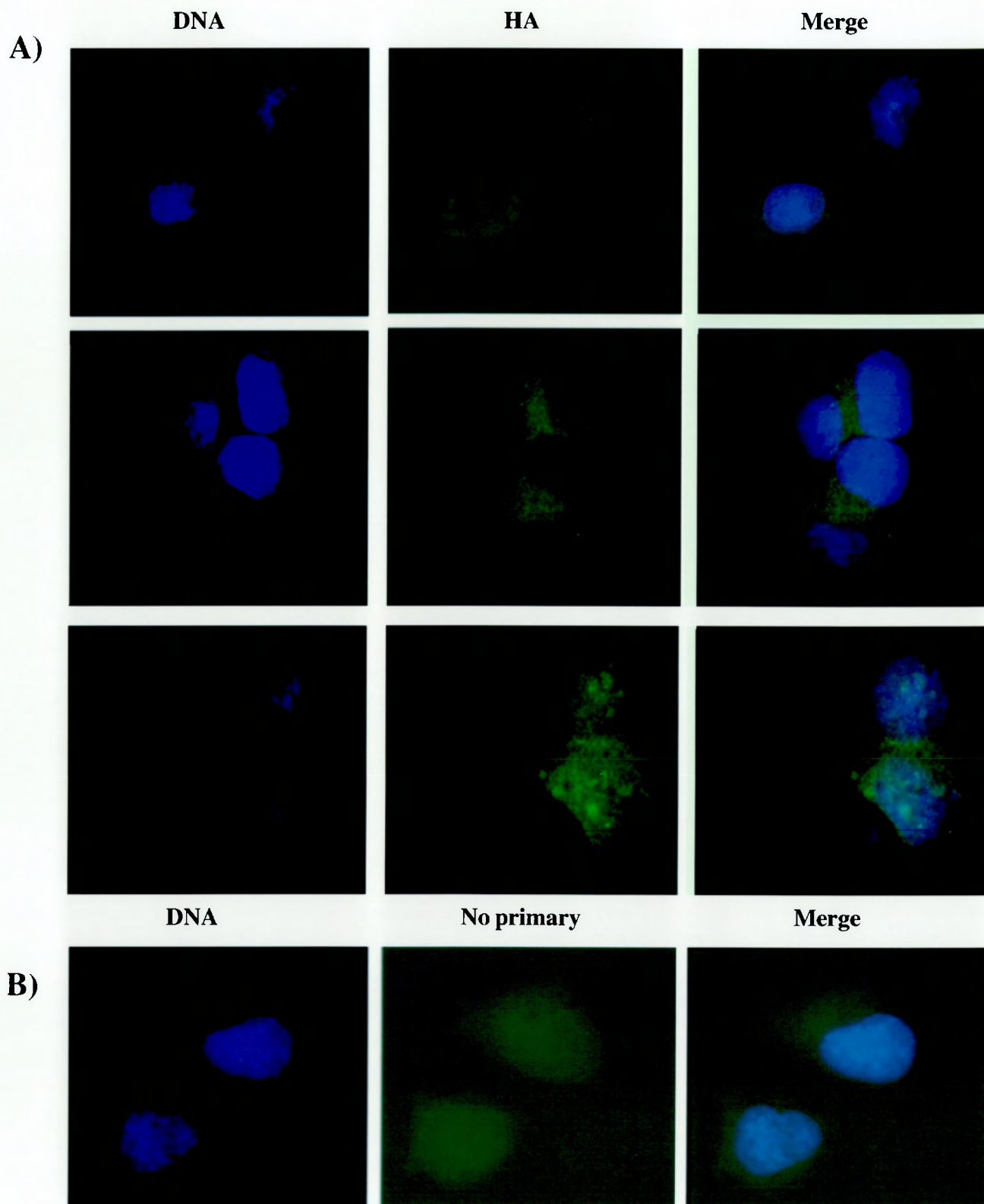


Figure 7.1 – Stably overexpressed HA-hPwp1 in COS-7 cells is localized throughout the nucleus and cytoplasm

Indirect immunofluorescence in COS-7 cells from polyclonal pool stably overexpressing HA-hPwp1. Cells were grown on sterile glass coverslips in a 6-multiwell plate for 24 hours and fixed in 4% paraformaldehyde, permeabilised in 0.5% Triton X-100. Cells were then stained using mouse monoclonal anti-HA antibody and FITC-conjugated anti-mouse antibody (A). As a negative control the cells were also stained with FITC-conjugated anti-mouse antibody alone (B). DNA was stained with Hoechst 33258 [1µg/ml] and the cells were then mounted in 90% glycerol and examined by fluorescence microscopy.

pEGFP-hPWP1 vector was then transiently expressed in COS-7 cells and its localization of the EGFP-Pwp1 fusion protein was detected by fluorescence microscopy. The protein was clearly found only in the cytoplasm, which contrasted with the nuclear and cytoplasmic localization described above (Figure 7.2).

The sequence of hPwp1 contains a putative nuclear localization signal (NLS) (Honore et al. 1994), which is also conserved in the budding and fission yeast proteins (Crane 2000; Duronio et al. 1992). Motifs of this sort allow proteins to enter the nuclear compartment of the cell by an energy-dependent mechanism involving β -importins (reviewed by Hodel et al. 2001). Given that the presence of this motif in a protein suggests its possible localization and function in the nuclear compartment of the cell, the exclusion of the EGFP-Pwp1 fusion protein from the nucleus was further elucidated. The fungal antibiotic leptomycin B (LMB) is able to inhibit the nuclear/cytoplasmic transport by its direct binding to Crm1, which is a highly conserved export factor (Adachi and Yanagida 1989; Fornerod et al. 1997a; Fornerod et al. 1997b; Ossareh-Nazari et al. 1997; Stade et al. 1997; Toda et al. 1992). This in turn affects the binding of Crm1 to the nuclear export signal (NES) sequence of cargo proteins, preventing their translocation from the nucleus to the cytoplasm and consequently leading to a nuclear accumulation of these proteins (Fornerod et al. 1997b; Kudo et al. 1998; Nishi et al. 1994; Ossareh-Nazari et al. 1997). The possibility that the cytoplasmic localization of EGFP-Pwp1 reflected rapid Crm1-dependent protein export was next addressed. To this end, COS-7 cells were transiently transfected with pEGFP-hPWP1 and 24 hours later were mock or LMB treated for 45 minutes or for 2.5 hours. DNA was stained using Hoechst 33285 and localization of the fusion protein was assayed by fluorescence microscopy. Figure 7.3 shows that treatment of the transiently transfected cells with leptomycin

B partially changed the localization of the EGFP-hPwp1 fusion protein. Panel A shows a cytoplasmic localization of the protein in the mock treated cells, whereas panel B (45 minutes) and panel C (2.5 hours) show that the fluorescence appears to be diffused throughout the cell. In conclusion, although exclusive nuclear localization of the protein was not observed after treatment with leptomycin B, the change of the localization pattern of the EGFP-hPwp1 suggests a possibly indirect but Crm1-dependent nuclear export mechanism.

HA-hPwp1 overexpression increases the activity of the AP-1 complex

Fission yeast *pwp1*⁺ is able to increase the transcriptional activity of Pap1 (Crane 2000) and the drug resistance conferred by Pwp1 is Pap1-dependent. Bearing in mind that the phenotypes conferred by the human and fission yeast genes are opposite, the possible role of human Pwp1 in the modulation of AP-1 activity in mammalian cells was explored.

Both transcriptional and DNA binding activities of the mammalian AP-1 complex were investigated. First, the DNA binding activity of AP-1 was measured in COS-7 polyclonal cells carrying the empty vector or overexpressing HA-hPwp1. Whole cell extracts were prepared from both cell populations and incubated for 30 minutes with a [³²P]-labelled double strand oligonucleotide containing the AP-1 DNA binding consensus sequence ATGACTCAG (Figure A.1 - *Appendix*). Free DNA labelled-oligonucleotides and DNA/AP-1 complexes were then separated by non-denaturing acrylamide electrophoresis. In competition experiments the samples were incubated with a 50-fold excess of unlabelled AP-1 consensus or mutant oligonucleotide (in which the consensus nucleotides CA are changed to TG) for 20 minutes before adding the labelled probe. As shown in the

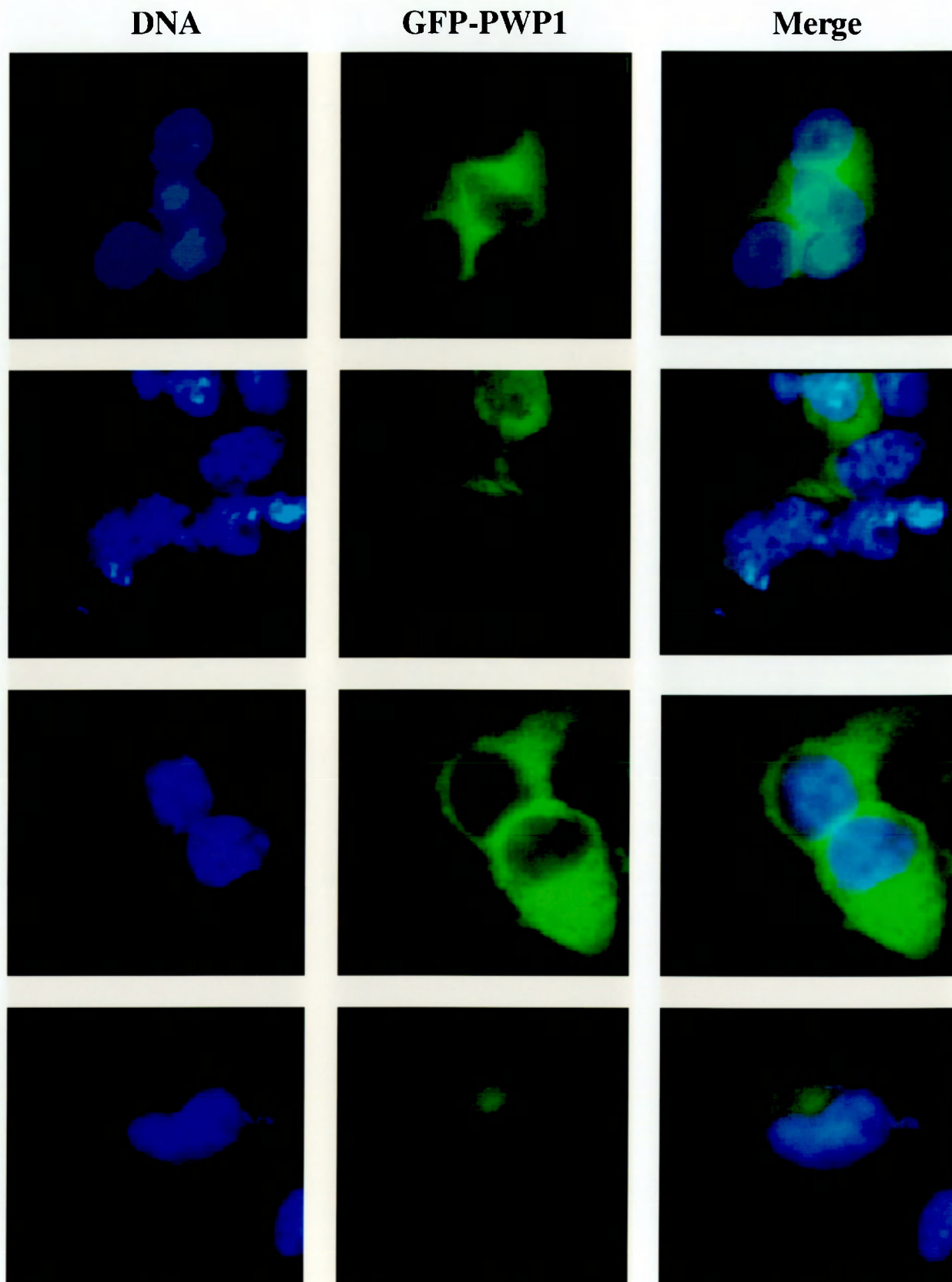


Figure 7.2 – Transiently expressed EGFP-Pwp1 is cytoplasmic

Localization of EGFP-hPwp in COS7 cells. Cells were grown on sterile glass coverslips in a 6-multiwell plate. Transient transfection of the pEGFP/hPWP1 plasmid was carried out using FuGENE reagent and 24 hours later the cells were fixed with 4% paraformaldehyde. DNA was stained with Hoechst 33258 [1µg/ml] and localization of EGFP-hPwp1 was detected by fluorescence microscopy.

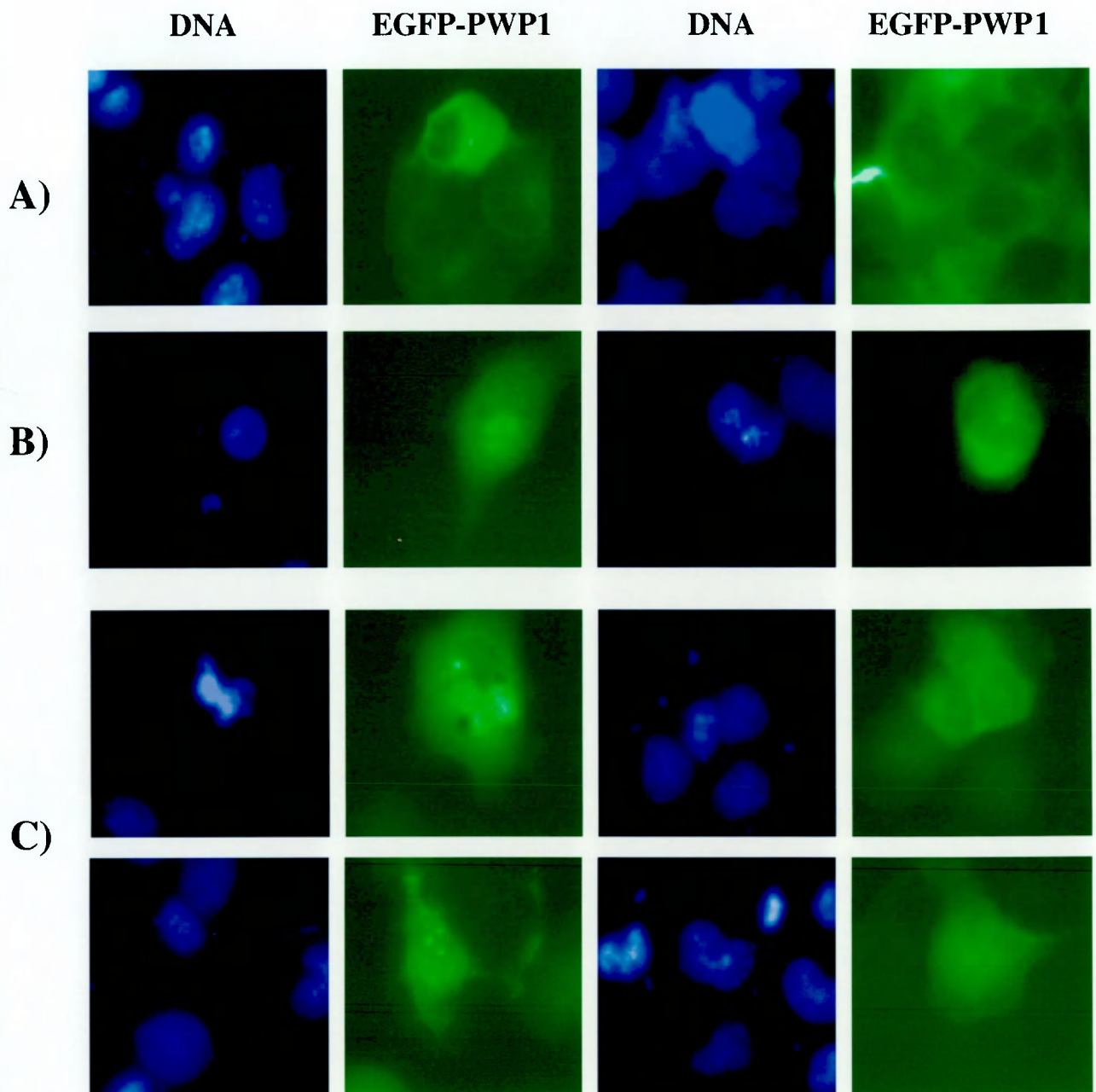


Figure 7.3 – Treatment with leptomycin B partially changes the localization pattern of transiently expressed EGFP-hPwp1

COS7 cells were grown on sterile glass coverslips in a 6-multiwell plate. Transient transfection of the pEGFP/hPWP1 vector was carried out using FuGENE reagent and 24 hours later the cells were mock (A) or leptomycin B treated for 45 minutes (B) or 2.5 hours (C). Cells were then fixed with 4% paraformaldehyde and DNA was stained with Hoechst 33258 [1µg/ml]. Localization of EGFP-hPwp1 was detected by fluorescence microscopy.

Figure 7.4, exogenous HA-hPwp1 increased the formation of DNA/AP-1 complex in COS-7 cells. The detected complex was AP-1 specific since its formation was abolished when an excess of unlabelled AP-1 consensus oligonucleotide was added to the samples.

In contrast, the presence of an excess of AP-1 mutant oligonucleotide did not affect the AP-1/DNA band-shift.

Next, the transcriptional activity of AP-1 was analysed using a luciferase reporter assay. COS-7 polyclonal cells carrying the empty vector or overexpressing HA-hPwp1 were transfected with the reporter pCol3Luc plasmid, expressing firefly luciferase under the control of the collagenase promoter (kindly provided by Dr. J.Tavare). This promoter contains a 9-bp consensus AP-1 DNA sequence and is modulated by AP-1 (Angel et al. 1987a; Angel et al. 1987b). The control pRL-TK-*Renilla* plasmid, which expresses *Renilla* luciferase under control of the constitutive thymidine kinase (TK) promoter, was co-transfected at a ratio of 15:1, as an internal control for transfection efficiency. The activity of both firefly and *Renilla* luciferases was measured using the dual luciferase reporter assay kit (Promega) and the results were expressed as the ratio between the firefly and the *Renilla* activities, referred as the relative luciferase activity (RLA). hPwp1-overexpressing cells had an AP-1 transcriptional activity 3-fold (A) or 5-fold (B) higher than cells carrying the empty vector, as observed in two independent experiments (Figure 7.5).

In conclusion, overexpression of human PWP1 in mammalian cells was able to increase both DNA binding and transcriptional activities of AP-1 transcription factors. This evidence is in accord with the Pap1 transcriptional activation observed in fission yeast after overexpression of Sppwp1⁺ (Crane 2000).

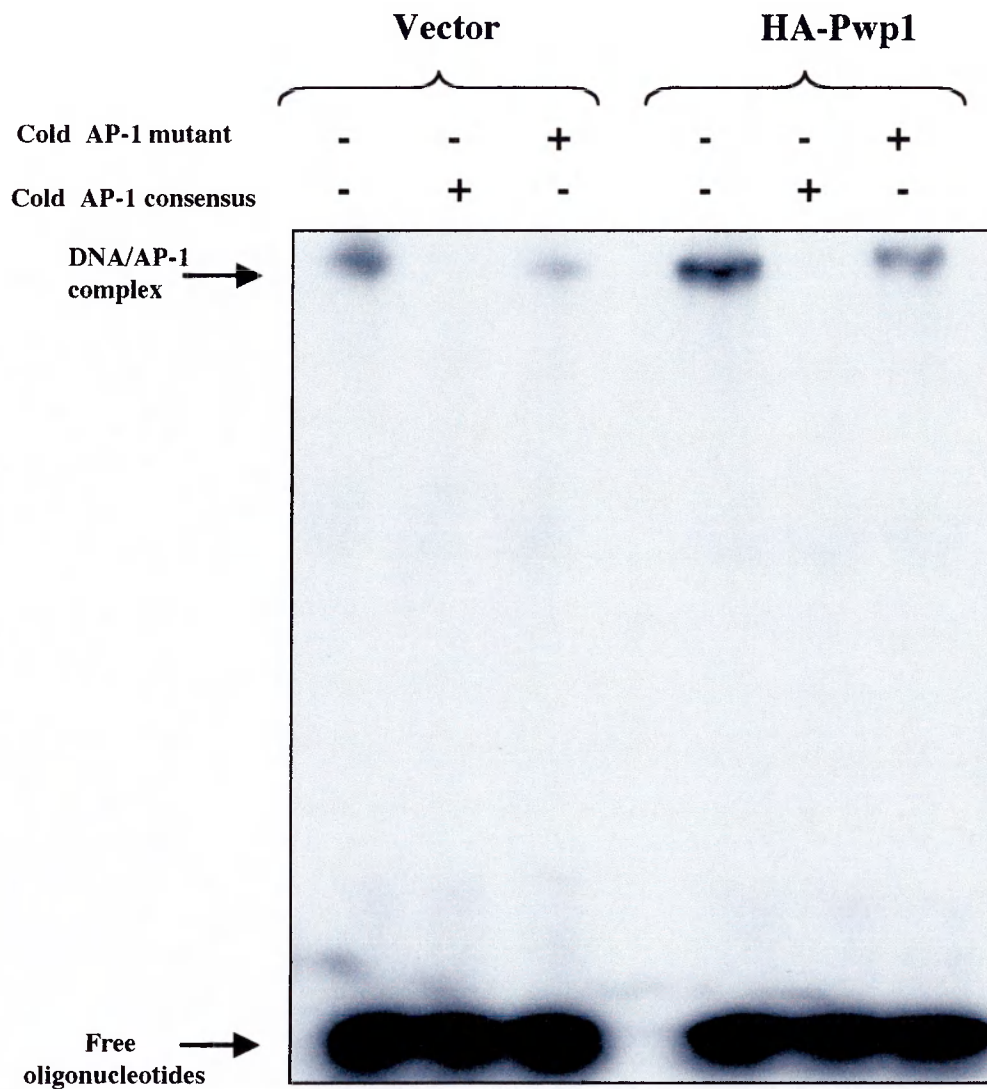


Figure 7.4 – Overexpression of HA-hPwp1 increases AP-1 DNA binding activity

AP-1 DNA binding activity was performed by electrophoretic mobility shift assay using whole cell extracts (WCE) prepared from polyclonal cell population stably transfected with the empty vector (pcDNA3.1+) or pcDNA3/HA-hPWP1. For competition experiments, 50-fold excess of unlabelled (“cold”) AP-1 consensus or mutant oligonucleotides was added (+) to the binding reaction for 20 minutes before adding the labelled AP-1 consensus probe.

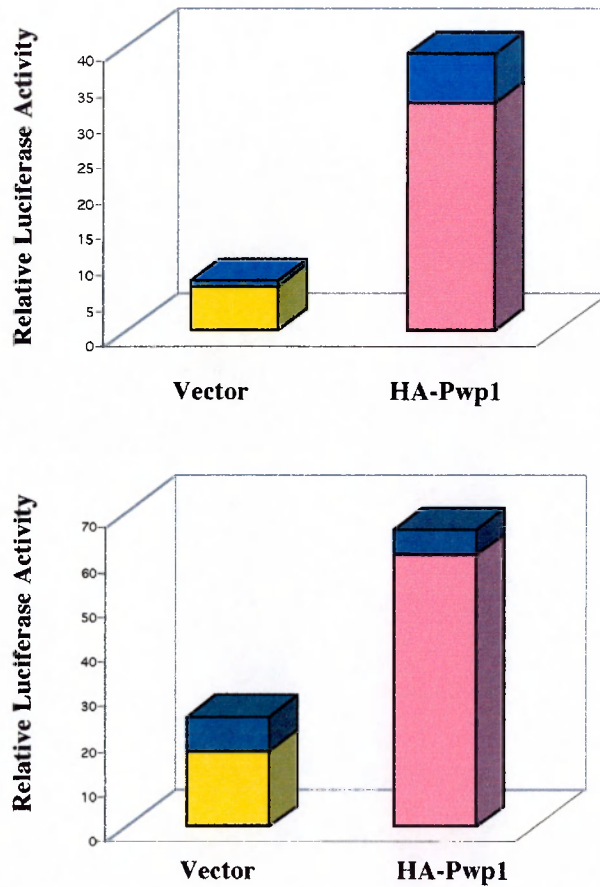


Figure 7.5 – AP-1 transcriptional activity is increased by overexpression of HA-hPwp1

Polyclonal pool COS-7 cells stably transfected with the pcDNA3.1/HA-hPWP1 vector or the empty vector were transiently co-transfected with pCol3-Luc and pRL-TK-Renilla plasmids at a ratio of 15:1. The cells were then assayed for the activities of both firefly and *Renilla* luciferase using the dual luciferase reporter kit (Promega). The results are expressed as relative luciferase activity (RLA) which is the ratio between the firefly and the *Renilla* activities. Standard deviation is shown as a dark blue shading. Two independent experiments are shown.

Anti-sense RNA expression strategy

Fission yeast *pwp1⁺* is essential an essential gene (Crane 2000). To this end and in order to investigate if decreased level of endogenous hPwp1 expression might lead to drug resistance, an anti-sense RNA strategy was chosen (Figure 7.6). hPWP1 cDNA was excised from pcDNA3.1+/hPWP1 and was cloned into opposite orientation into the pcDNA3.1- vector (InVitrogen), whose multicloning site is in the opposite orientation to that of pcDNA3.1+. The resulting plasmid was named pcDNA3.1-/PWP1-AS. This plasmid should direct expression from the anti-sense strand of *hPWP1*. pcDNA3.1-/PWP1-AS was stably transfected into COS-7 cells and after selection polyclonal G-418-resistant cells were obtained. 6 different stable clones were also derived from this polyclonal pool.

The cells transfected with anti-sense hPWP1 grew slowly in comparison with untransfected cells, empty vector or hPWP1-transfected cells. This slow growth phenotype was reminiscent of that observed in *S.cerevisiae* *PWP1*-deleted cells (Duronio et al. 1992). In order to investigate this phenotype in more detail growth kinetics were determined (Figure 7.7). The growth of COS-7 polyclonal cells carrying the empty vector was compared with that of polyclonal (A) and clonal (B) cells expressing anti-sense hPWP1. Equal numbers of cells were plated in duplicate in a 6-multiwell plate and the following day the cells were harvested (day 0) and counted using a haemocytometer. The cells were then counted at days 1, 3, 5 and 7 in the experiment in panel A and at days 2, 7 and 9 in the experiment in panel B. The results show that cells stably expressing the anti-sense hPwp1 had a doubling time approximately twice that of cells carrying the empty vector.

In parallel, the cell cycle profile in these cells was analysed by flow cytometry, but no major differences in the cell cycle phase distribution among cells carrying

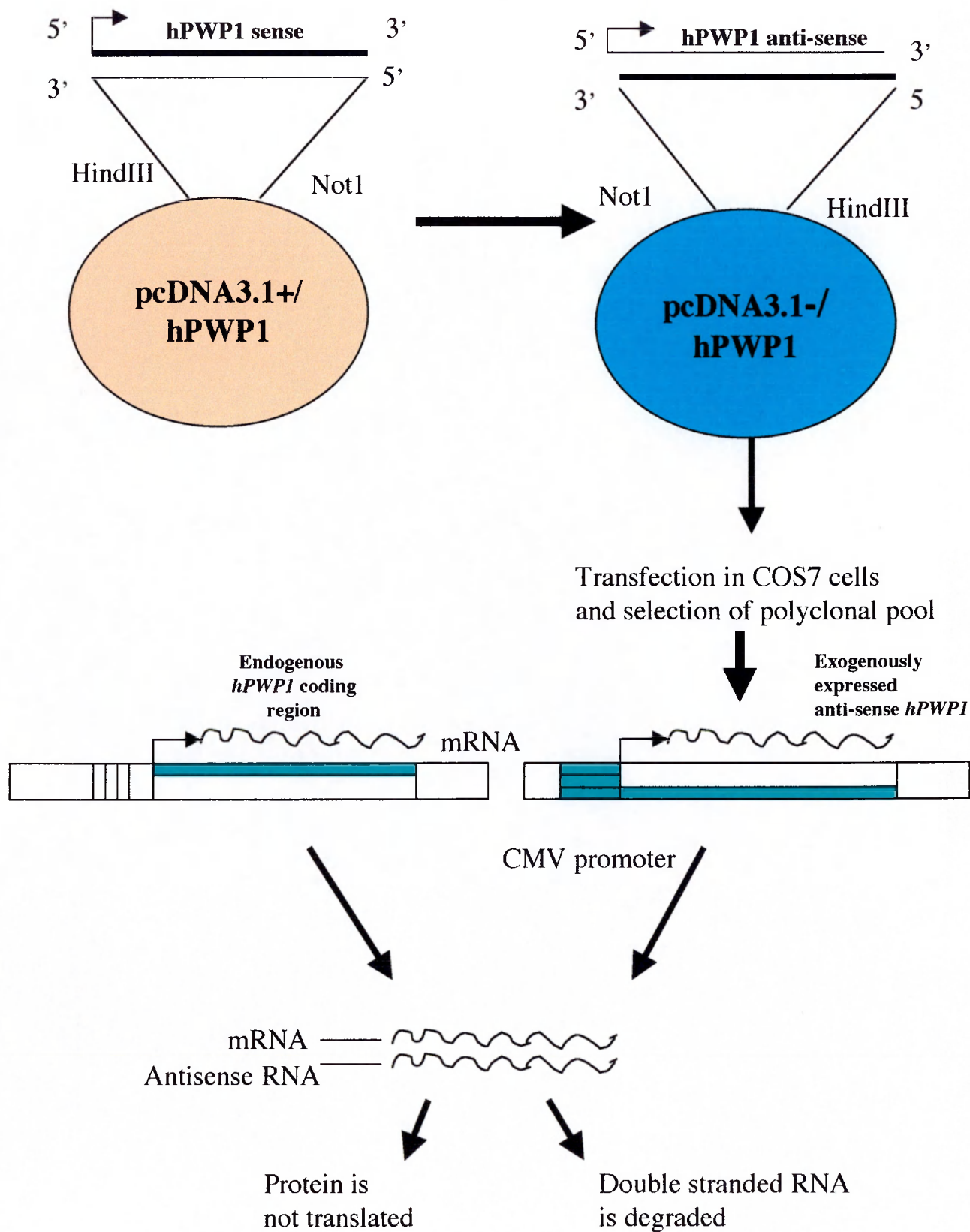


Figure 7.6 – The RNA anti-sense strategy

the vector, polyclonal cells expressing HA-hPwp1 or anti-sense hPwp1 were observed (data not shown).

A possible explanation for this slow growth phenotype might be that the frequency of apoptosis was somehow increased by reduction of hPwp1 expression. To address this hypothesis, the percentage of apoptotic or preapoptotic cells was measured in anti-sense hPwp1 cells and cells carrying the empty vector using an Annexin V/propidium staining method (see Chapter 2). However, no significant difference between the two cell populations was found (data not shown).

These results indicate that neither increased apoptosis nor cell cycle-specific defect was responsible for slow growth seen in anti-sense hPwp1 cells.

The nuclei of anti-sense Pwp1 cells were also observed by microscopy with the aim of identifying any possible abnormality at the nuclear level. The cells from a polyclonal pool carrying the empty vector or expressing anti-sense Pwp1 were grown on glass coverslips in a 6-multi-well plate for 24 hours. Then, the nuclei were stained using Hoechst and analysed by microscopy. The Figure 7.8 shows that about 20% of the anti-sense hPwp1 cells have fragmented nuclei versus 5% found in cells carrying the empty vector.

Human Pwp1 and interacting partners

Given that Pwp1 belongs to the WD-40 repeat family (Duronio et al. 1992) and that the WD-repeat motif is thought to mediate protein-protein interactions (Neer et al. 1994; van der Voorn and Ploegh 1992), the size of complex fractions containing hPwp1 was investigated. To this end, the proteins from the COS-7 polyclonal cells expressing HA-hPwp1 were separated by 7-37% sucrose density

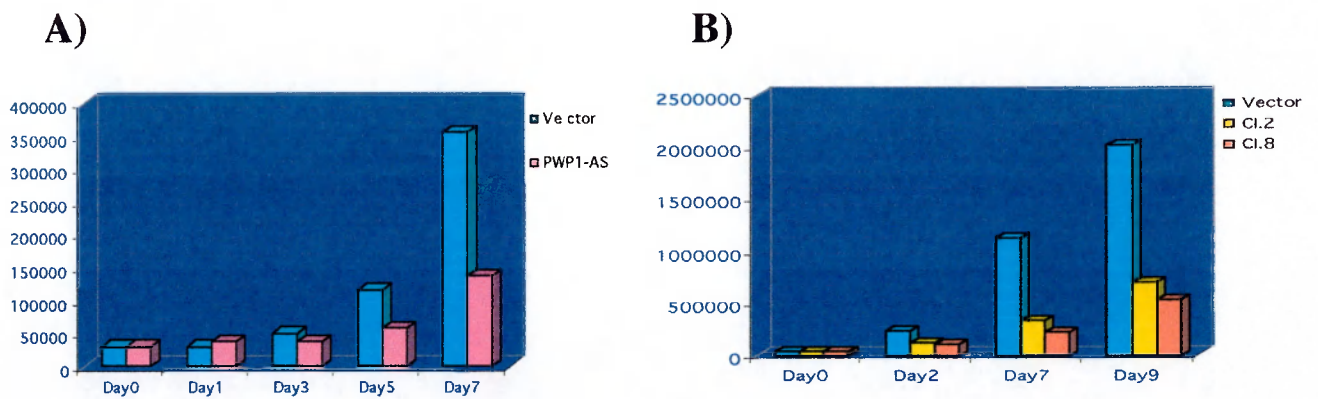


Figure 7.7 – Anti-sense Pwp1 cells have a slow growth phenotype

The growth rates of polyclonal COS-7 cells carrying the empty vector (pcDNA3.1+), polyclonal (A) and two clones (B) of COS-7 cells stably transfected with pcDNA3.1-/PWP1-AS were determined. The number of the cells in each population were counted using a haemocytometer at days 0, 1, 3, 5 and 7 (A) or at days 0, 2, 7 and 9 (B).

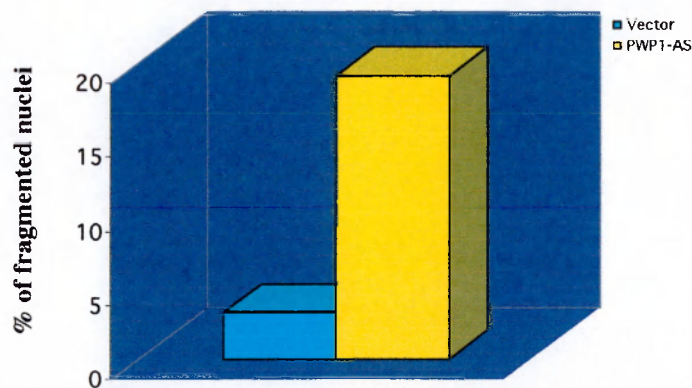
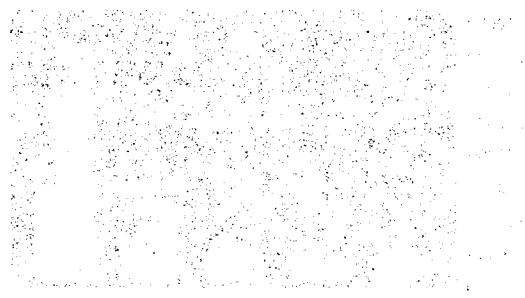
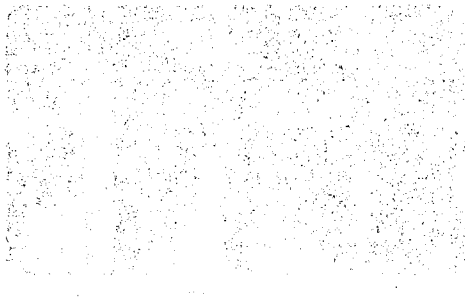
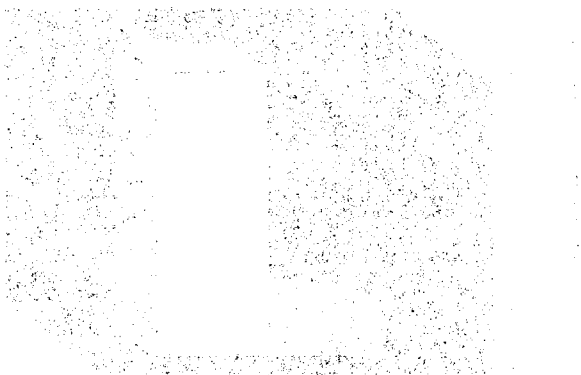


Figure 7.8 – Anti-sense Pwp1 cells have a high percentage of fragmented nuclei

Polyclonal pools carrying the empty vector or anti-sense hPwp1 were grown on sterile glass cover-slips in a 6-multiwell plate. 24 hours later the cells were fixed with 4% paraformaldehyde and the nuclei were stained with Hoechst 33258 [1mg/ml]. The nuclei were observed by microscopy and counted and the number of fragmented nuclei is expressed as the percentage of the total counted.



The first of these is the fact that the object is not a simple rectangle. It has a complex shape, with a large, irregular opening in the center. This opening is not a simple circle or oval, but a more complex, irregular shape. The second fact is that the object is not a simple rectangle. It has a complex shape, with a large, irregular opening in the center. This opening is not a simple circle or oval, but a more complex, irregular shape.



The third fact is that the object is not a simple rectangle. It has a complex shape, with a large, irregular opening in the center. This opening is not a simple circle or oval, but a more complex, irregular shape. The fourth fact is that the object is not a simple rectangle. It has a complex shape, with a large, irregular opening in the center. This opening is not a simple circle or oval, but a more complex, irregular shape.

gradient centrifugation (by R.Murray). An aliquot of each fraction was subjected to immunoblot analysis using HRP-conjugated monoclonal anti-HA antibody. (data not shown). HA-hPwp1 was detected mainly in fractions 1 and 2, which contain the smallest size complexes. This result suggests that hPwp1 is possibly part of a complex with an estimated size of approximately 300 kDa molecular mass.

On the basis of these results, a yeast two-hybrid screen was next performed in order to identify putative hPwp1 interacting proteins, which might in turn shed light on the possible functions of Pwp1 in the cell.

To generate the two-hybrid "bait", human *PWPI* cDNA was cloned in frame with the DNA binding protein LexA in the vector pEG202 and transformed into *S.cerevisiae* EGY48 cells. These cells contain two *LexA* operator-responsive reporters. One is a chromosomally integrated copy of the *LEU2* gene and the second is a plasmid bearing the *GAL1* promoter-*LacZ*, which causes the yeast cells to turn blue on agar containing X-gal.

The main requirements for a bait protein are firstly that it should not be actively excluded from the yeast nucleus and secondly that it should not possess an intrinsic ability to strongly activate gene transcription (Ausubel et al.). The latter requirement was examined for hPwp1. The empty pJG4-5 vector, used to clone the human cDNA library, was transformed into EGY48-Pwp1 cells and the vector-containing cells were selected on agar plates lacking uracil and histidine. A β -galactosidase assay was then performed and no blue colonies were identified, suggesting that hPwp1 cannot induce gene expression on its own. Whether the protein acts as a co-activator of transcription still remains to be investigated.

Next, an interaction hunt was performed. The sequences from the cDNA library were cloned under control of GAL1 galactose-inducible promoter in the pJG4-5 vector. After transformation of the human cDNA library into yeast cells containing the LexA-hPwp1 fusion protein bait and the two LexA reporters, if a protein X encoded by the cDNA library is able to interact with the LexA-Pwp1 fusion protein, which is bound to the LexA-responsive element, gene transcription will be activated. In contrast, if no interaction occurs there should be no transcription of the reporter genes (Figure 7.9).

Human HeLa cDNA library was transformed into the EGY48-Pwp1 yeast cells that were then selected on agar plates lacking histidine, uracil and tryptophane. β -galactosidase assays were then carried out in order to detect any positive interaction between hPwp1 and proteins encoded by the library plasmids. In two independent experiments an average of 15 colonies that became blue after incubation with X-Gal at 30°C were identified and isolated. The signal was never strong and furthermore always appeared 24-36 hours after the incubation with X-Gal. The cDNA-containing plasmid was rescued from each of the colonies obtained in the first screen and subjected to sequencing analysis. Three sequences only were obtained: empty vector, annexin II and cytochrome oxidase. Unfortunately, each of those sequences was represented in a list of the most common false positive sequences (<http://www.fccc.edu/research/labs/golemis.intro.html>) found in the yeast two-hybrid screens. To further check if these cDNAs were false positives, they were re-transformed into EGY48-Pwp1 yeast cells but no signal was observed, indicative of no interaction.

Unfortunately, no protein(s) interacting with hPwp1 were identified and the only conclusion drawn was that hPwp1 does not seem to be a transcription factor. More efforts are necessary in order to identify any putative partner interacting with the human Pwp1

Discussion

It is here shown that human Pwp1, which is localised in both the cytoplasmic and nuclear compartments of COS-7 cells, is able to positively modulate the activity of the AP-1 transcription factors. It is still not clear if hPWP1 is an essential gene; however the results from the anti-sense strategy suggest a role for hPwp1 in the regulation of cell growth. In addition, the protein seems to be part of low molecular weight complexes but unfortunately no interacting protein(s) has been identified so far.

hPwp1 and AP-1 transcription factors

The ability of human Pwp1 to positively modulate both DNA binding and transcriptional activity of AP-1 is in accord with the evidence that overexpressed *S.pombe pwp1*⁺ is able to increase the level of p25 protein level (Crane 2000), encoded by the Pap1-target gene *apt1*⁺ (Toda et al. 1992), which is indicative of an elevated activity of the AP-1-like transcription factor Pap1. However, there are no data regarding a possible modulation of Pap1 DNA binding activity after overexpression of fission yeast *pwp1*⁺ (Crane 2000).

These results taken together may suggest that the increased sensitivity observed in COS-7 cells overexpressing human Pwp1 after exposure to cytotoxic drugs (described in Chapter 6) could reflect elevated AP-1 activity which in turn leads to transcriptional activation of downstream targets.

It seems that although overexpression of fission yeast *pwp1*⁺ causes multi-drug resistance (Crane 2000), whereas overexpressed human *PWPI* leads to multi-drug sensitivity (Chapter 6), the general mechanism through which these proteins

respond to cytotoxic insults has been conserved. If this is the case, then a diversification of the downstream response triggered by AP-1/Pap1 activation must have occurred between yeast and human cells during evolution.

Elevation of apoptosis might cause survival disadvantage after treatment with cytotoxic drugs via the AP-1 transcription factors. AP-1 activity has been implicated in the regulation of a variety of cellular processes (reviewed by (Wisdom 1999; Wisdom et al. 1999). In fact, AP-1 has a key role in the regulation of cell proliferation and growth and it has also a protective role from the UV-induced cell death (reviewed by Karin et al. 1997; Wisdom 1999). A strong and prolonged induction of c-Jun has been also reported in consequence of stress inducing stimuli, including UV and ionising radiation, hydrogen peroxide and tumour necrosis factor α (TNF α), which can trigger apoptosis (see Bossy-Wetzel et al. 1997). Although it seems a paradox, AP-1 plays a role also in apoptosis. Cell death induced by growth factors withdrawal or by genotoxic agents, such as UV and x-ray, is mediated by AP-1 activity (Colotta et al. 1992; Gillardon et al. 1994; Goldstone and Lavin 1994) as well as c-Jun transcriptional activity is also required to trigger apoptosis in immortalized fibroblasts (Bossy-Wetzel et al. 1997).

This body of information together with the evidence discussed above can suggest that the Pwp1-induced cell sensitivity in response to cytotoxic stress might be mediated by AP-1 which in turn trigger apoptosis via transcriptional activation of cell death factors or of inhibitors of cell proliferation or of survival promoting factors.

HA-hPwp1 sub-cellular localization

In regard to the sub-cellular localization Honore' et al. previously concluded that human Pwp1 was more abundant in the nucleus of human cells than in the cytoplasm (Honore et al. 1994). In accord with that, I showed that exogenous stably expressed HA-tagged Pwp1 was localised both in the cytoplasm and nucleus of COS-7 cells, with a stronger signal in the nucleus. By comparison, transiently expressed EGFP-tagged Pwp1 was excluded from the nucleus. Given the fact that the Pwp1 amino acid sequence contains a putative nuclear localization signal (KKKGK (Duronio et al. 1992)), it seems likely that the protein is able to enter into the nuclear compartment through the importin β -dependent mechanism via active transport. As discussed for the localization of HA-Poh1 in Chapter 3, the EGFP-epitope tag fused to the amino-terminus of hPwp1 could modify the conformation of the protein with consequent inhibition of its nuclear translocation. Alternatively, the different localization pattern observed for the HA-Pwp1 and EGFP-Pwp1 proteins might simply reflect to a stable expression of the first compared to transient expression of the second, which could give rise to artefacts. Treatment of cells transiently overexpressing EGFP-Pwp1 with leptomycin B resulted in re-distribution of the protein from the cytoplasm to the entire cell. This indicates on the one hand that EGFP-tagged hPwp1 is also able to enter into the nucleus and on the other that the localization of hPwp1 might be modulated at the level of nuclear export, as leptomycin B inhibits nuclear/cytoplasmic traffic dependent on Crm1 export factor (Fornerod et al. 1997b; Kudo et al. 1998; Nishi et al. 1994; Ossareh-Nazari et al. 1997). There is no readily identifiable nuclear export signal (NES) in the Pwp1 sequence,

however the nuclear/cytoplasmic trafficking could be regulated indirectly, presumably mediated by a hPwp1 interacting protein.

hPwp1 and interacting proteins

No interaction partners for Pwp1 in different species have been previously identified. Here, I describe two halves of a same attempt aimed to identify a possible hPwp1 interacting partner using a two-hybrid screen analysis but unfortunately they were unsuccessful. Nevertheless, it was also shown that the protein is part of a small size complex. This data is not in accord with the evidence that fission yeast Pwp1 was found in fractions containing complexes with even larger size than polysomes after sucrose density gradient centrifugation (Crane 2000). This difference might further support the notion that the functions of *S.pombe pwp1*⁺ and human *PWP1* have not been conserved during evolution. In fact, the two counterparts are not functional homologues and furthermore when overexpressed they lead to opposite cell responses after the exposure of the cells to a given cytotoxic drug (Chapter 6), although they are both able to mediate their responses through Pap1/AP-1 transcription factors. Therefore, it is less surprising that the two proteins are also localised to different areas of the cell and are part of complexes of different sizes in the cell.

The RNA anti-sense strategy

The slow growth phenotype observed after human Pwp1 anti-sense RNA expression in COS-7 cells suggests a possible role of the protein in cell growth regulation, although the results did not fully answer the question if the human gene is essential. This evidence is in accord with the phenotype observed in

S.cerevisiae, where the *PWPI*-deletion mutants grow three-fold more slowly than wild-type cells, although the gene is not essential (Duronio et al. 1992). In contrast, *S.pombe pwp1*⁺ is an essential gene (Crane 2000). However, it was here not demonstrated a decreased level of the endogenous Pwp1 in COS-7 cells transfected with pcDNA3.1-/PWPI-AS as the anti-Pwp1 antibody was available only recently.

The retardation in the cell growth observed in hPwp1 anti-sense cells was not due to a specific cell cycle delay or to elevated apoptosis. In addition to this growth phenotype, the cells in culture appeared also generally unhealthy and the nuclei had a higher percentage of fragmentation compared to control cells. The significance of this phenotype and the mechanism by which stage the reduced level of endogenous Pwp1 in the cell, if any, could cause these defects need still to be elucidated.

The preliminary results described here together with a few lines of evidence, such as the phenotype observed in the *PWPI*-deleted mutants in *S.cerevisiae* (Duronio et al. 1992), the presence of highly charged residues in the amino acid sequence of Pwp1 (Duronio et al. 1992), and the regulatory role of each member of the WD-repeat family in the cellular processes (Neer et al. 1994), further support the hypothesis that hPwp1 might be involved on the regulation of cell growth or possibly of gene transcription (Duronio et al. 1992).

General conclusions and further directions

The work described in this thesis, concerning the characterization of novel human determinants of drug resistance in mammalian cells, highlights the key role played by transcriptional regulation in the cellular responses to cytotoxic insults, although the downstream responses can be diverse and even opposite.

During the course of this work it was established that at least some mechanistic aspects of multi-drug resistance mechanism are conserved between fission yeast and human cells. The highly conserved 26S proteasome regulatory subunit Poh1/Pad1 appears to function as transcriptional co-activator of the basic leucine zipper AP-1/Pap1 transcription factors when overexpressed. This evidence together with previous reports strongly suggests that Poh1 can regulate the drug susceptibility of cells via modulation of the ubiquitin-dependent proteolysis of transcription factors. However, the downstream effector mechanisms are still unidentified. Ongoing studies are aimed at identifying targets of the HA-Poh1-induced pleiotropic drug resistance mediated by AP-1 using a microarray transcriptional profiling approach and *HA-POH1* doxycyclin inducible human cells. In order to address the general function of Poh1 as regulatory subunit of the 26S proteasome and any dependency of Poh1-induced pleiotropic drug resistance on the 26S proteasome complex several aspects could be addressed. The interaction of exogenous HA-Poh1 with c-Jun (Figure 5.13) and the evidence that most HA-Poh1 is not incorporated into the 26S proteasome (Figure 3.10) may suggest a proteasome-independent mechanism. Purification of recombinant Poh1 would be useful for the development of *in vitro* assays that will allow exploration of the role of Poh1 in the recognition of ubiquitinated substrates, such as c-Jun and c-Fos, and their consequent proteolysis. It would be also valuable to test the

possibility that Poh1 acts as a ubiquitin hydrolase. The identification of additional Poh1 interacting proteins other than c-Jun using a two hybrid screen approach should be quite straightforward. Furthermore, although it was previously demonstrated that HA-Poh1 is able to functionally complement *pad1*-deleted mutants and to confer drug resistance (Spataro et al. 1997), the possibility that the HA epitope tag fused to the amino-terminus of the protein causes a disruption of Poh1 function and subsequently drug resistance has not yet been addressed. This may explain the differing abilities of the exogenous and endogenous Poh1 to associate with the 26S proteasome complex and to interact with c-Jun. Studying the drug susceptibilities of the cells overexpressing Poh1 or HA-Poh1 in parallel could be a simple way to address this topic. Given that proteolysis defects associated with growth defects have been reported in the fission yeast temperature sensitive *pad1-1* mutant (Penney et al. 1998), it would be also interesting using the anti-sense RNA strategy to examine the effects of reduction of Poh1 protein level on ubiquitin-dependent proteolysis and growth. At the same time, it will be interesting to determine whether a reduced level of Poh1 confer drug sensitivity.

PWPI was shown to confer multi-drug sensitivity in mammalian cells and to activate AP-1 activities when overexpressed, suggesting transcriptional activation of proteins involved in apoptosis or in the inhibition of cell survival. Earlier speculations on the function of Pwp1 led to the hypothesis that Pwp1 might be involved in cell growth and/or transcriptional regulation. Many of the questions addressed in this study are still unsolved and to elucidate the possible mechanism through which Pwp1 affects drug sensitivity it will be necessary to focus more on the normal function of Pwp1 in the cell.

First, it will be important to identify Pwp1-interacting proteins in order to know more about the Pwp1-containing complex and its possible function in the cell. The previous attempts using a two-hybrid screen analysis were unsuccessful possibly because more colonies need to be screened. The identification of downstream targets of the AP-1 activation induced by Pwp1 using microarray transcriptional profiling analysis, as for Poh1, would shed light on the mechanism underlying the Pwp1-induced drug sensitivity of the cells. Because of the late availability of the anti-Pwp1 antibody and consequent lack of time it was not possible to determine whether the slow growth phenotype observed in the anti-sense Pwp1 COS-7 cells is strictly due to a reduced expression level of Pwp1. Such an experiment is now possible and would be an early priority in the extension of the work described here.

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Appendix

A.1) Sequences

- Oligonucleotide primers

ICRF n	Name	Sequence
=====		
1	94947	PWP1 5'
2	94948	PWP1 3'
3	1122113	PWP1 <i>Hind/Sa</i>
4	1122114	PWP1 (3HA)
5	105066	PWP1 <i>Hind</i> HA3
6	(Richard)	PWP1ATG (no Tag)
7	97356	PWP1 mutchk
8	97358	PWP1 3'Pst
9	115202	PWP1 5'int
10	101911	RETOFF 5'
11	124795	RETOFF 3'
12	124795	Linker 5'
13	124796	Linker 3'
14	116171	POH1 <i>Sal</i> 5'
15	116172	POH1 <i>Xma</i> 3'
17	1244868	POH1 <i>EcoRI</i> 3'
18	1244869	POH1 <i>NotI</i> 5'
19	83047	PJG45X
20	83048	PJG45E

- AP-1 oligonucleotides

AP-1 consensus	5'-cgc ttg atg act <u>cag</u> ccg gaa-3' 3'-gcg aac tca tga gtc ggc ctt-5'	Santa Cruz (<i>sc-2501</i>)
AP-1 mutant	5'-cgc ttg atg act <u>tgg</u> ccg gaa-3' 3'-gcg aac tac tga acc ggc ctt-5'	Santa Cruz (<i>sc-2514</i>)

- Peptides

PWP1-NT	KETPDKVELSKEEVKR	189 mg
PWP1-CT	SRSSDTPME	77 mg

A.2) Vectors

- Vectors used for cloning

Name	Description
pREP3	Fission yeast expression vector
pcDNA 3.1+/-	Constitutive expression
pRETRO OFF	Tetracycline repressible expression
pOPRSVI CAT	IPTG-inducible expression
pGEX-4T-3	GST-fusion bacterial expression
pUHD10-3MCS	Tetracycline/doxycyclin regulatable expression
pIRES2-GFP	GFP expression
pEGFP-C1	GFP-fusion protein expression
pEG202	LexA-fusion for yeast two hybrid screen

- Other plasmids

Name	Description

pCD2/CMV	Transient co-transfection and CD2 selection
pCol-LUC3	Luciferase assay
pRL-TK-Renilla	Luciferase assay
pcDNA/HA-Ub	c-Jun/c-Fos ubiquitinylation
pMT35/cJun-His ₆	c-Jun ubiquitinylation
pcDNA1/ c-Jun ₁₋₂₈₇	c-Jun <i>in vitro</i> degradation assay
pcDNA1/c-Jun Δ ₃₋₁₂₂	c-Jun <i>in vitro</i> degradation assay
pcDNA1/c-Jun dominant neg.	Transient transfection for drug sensitivity

A.4) Antibodies

- Primary

Antibody	Source	Type	Concentration/ Dilution
HA-peroxidase high affinity (3F10)	Roche	rat monoclonal antibody	12.5 ng/ml
Flag M2	Sigma	Mouse monoclonal	1:1000
Hap-1	The Binding site	Sheep polyclonal	1:1000
c-Jun (H-79) [200 µg/ml]	Santa Cruz Biotechnology	rabbit polyclonal antibody	1:50 IP 1:100 WB
c-Jun/AP-1 (Ab-1) [100 µg/ml]	Clontech	rabbit polyclonal antibody	1:50 IP 1:100 WB
c-Fos (4) [200 µg/ml]	Santa Cruz Biotechnology	rabbit polyclonal antibody	1:100
MCP-21 [1.9 µg/µl]	ICRF Research Monoclonal Antibody service	mouse monoclonal antibody	1:500 IP 1:1000 WB
CD2 OX-34 [0.3 µg/µl]	Serotec	mouse monoclonal antibody	2 µg/ml
Cdc2 (A17)	G. Gammon and T. Hunt	mouse monoclonal	2 µg/ml
Poh1 CN13	Spataro et al 1997	rabbit polyclonal antibody	1:200
GFP B34 [1mg/ml]	Covance	murine monoclonal antibody	1:1000

HA HA.11 (16B12)	Covance	mouse monoclonal antibody	1:200 or 1:500 IF
Pwp1 CN35	This study	rabbit polyclonal	1:200
c-Jun/AP-1 (N)X 200 µg/0.1 ml	Santa Cruz Biotechno nogy		4-8 µg/ml

- Secondary

Antibody	Source	Dilution
anti-rabbit peroxidase conjugates	Sigma	1:1000 or 1:2000
Anti-sheep peroxidase conjugated	Sigma	1:1000
anti-mouse peroxidase conjugates	Sigma	1:1000 or 1:2000
anti-mouse FITC- conjugated	Sigma	1:800

Overview

In this chapter I describe the molecular mechanism by which overexpressed HA-Poh1 leads to pleiotropic drug resistance in mammalian cells.

Firstly, I introduce AP-1 transcription factors, the links between AP-1 and the 26S proteasome complex and drug resistance in human cells. Secondly, I describe the ability of HA-Poh1 to positively modulate AP-1 transcription factor activity associated with increased protein levels of the two AP-1 subunits, c-Jun and c-Fos. Furthermore, evidence is presented that HA-Poh1 may increase the protein stability of c-Jun through an interaction between the two proteins.

Introduction

Activating protein 1 (AP-1)

Activating protein 1 (AP-1) is a family of dimeric transcription factors complex, whose components include c-Jun and c-Fos, members of the bZip family proteins which contain conserved basic (b) and leucine zipper (ZIP) domains required for their binding to DNA. This motif mediates homodimerization of c-Jun and heterodimerization of c-Jun and c-Fos and the subsequent recognition and binding to the palindromic consensus DNA sequence 5'-TGAGTCA-3', referred as 12-*O*-Tetradecanoylphorbol 13-acetate (TPA)-responsive element (TRE) or AP-1 consensus site, located in the promoter region of a variety of genes (Angel et al. 1987b). c-Jun and c-Fos are both defined proto-oncogenes as they were identified as the cellular counterparts of the oncogenes v-Jun and v-Fos, respectively (Maki et al. 1987) (Curran and Teich 1982). c-Jun is also able to

form heterodimeric complexes with ATF-2 (Activating Transcription Factor 2), belonging to the ATF transcription factor family, and CREB proteins. The resulting complexes bind to the AP-1-related cyclic AMP responsive element (CRE)-like site 5'-TGAGCTCA-3' (reviewed by Karin et al. 1997; Wisdom 1999).

A wide range of external stimuli, such as peptide growth factors, cytokines, oxidative stress, UV irradiation and other forms of cellular stress, generate signals that converge on the AP-1 factors (Figure 5.1), with subsequent modulation of their activity which is regulated primarily at the transcriptional and post-translational levels (Figure 5.2). Based on this regulation, AP-1 co-ordinates a variety of signals and consequently generates diverse cellular responses, including cell proliferation, differentiation, development, apoptosis and other stress responses (reviewed by Karin et al. 1997; Wisdom 1999) (Figure 5.1).

DNA binding and transcriptional activation domains are distinct in c-Jun and c-Fos and the activation domains are modulated by phosphorylation. For example, c-Jun is phosphorylated at Ser⁶³, Ser⁷³, Thr⁹¹ and Thr⁹³ by members of the c-Jun NH₂-terminal kinases (JNK) family (Adler et al. 1994). JNK, originally identified as a c-Jun associated kinase, is a serine/threonine kinase activated by phosphorylation by various stimuli, including DNA damaging agents, inhibitors of protein synthesis, heat shock and osmotic stresses (reviewed by Kyriakis et al. 1994). Phosphorylation of c-Jun controls its activity in two manners (Figure 5.2). On the one hand, the tight association between JNK and c-Jun, which is independent on the activation status of JNK, targets c-Jun for ubiquitinylation and on the other hand phosphorylation at Ser⁷³ by JNK is sufficient to protect the protein from ubiquitinylation resulting in increased protein stabilization (Fuchs et al. 1997) (Figure 5.3). This probably occurs through a conformational change of c-Jun after phosphorylation which consequently affects its affinity for JNK.

A few lines of evidence have demonstrated that in fibroblasts the mitogenic response of the cell is mediated through AP-1 activity, resulting in modulation of cell growth and proliferation (Brown et al. 1998; Kovary and Bravo 1991; Maki et al. 1987). c-Jun and c-Fos play also a major role in the cellular response to UV irradiation by protecting the cell from UV-induced cell death (Wisdom, 1999) (Schreiber et al. 1995). It was recently reported that c-Jun modulates cell proliferation and UV responses by distinct mechanisms, both involving modulation of the transcription of the cyclin D1 gene (Wisdom, 1999) (Wisdom et al. 1999). Regulation of cell proliferation does not require c-Jun phosphorylation, whereas phosphorylation of c-Jun at Ser⁶³ and Ser⁷³ sites with subsequent activation of the protein is necessary to protect the cell from UV-induced cell death.

c-Jun degradation pathways

c-Jun is a short-lived protein with a half-life of approximately 90 minutes (Treier et al. 1994). Tight temporal control of the activity of many regulators of gene transcription, including c-Fos, p53, c-Mos and c-Myc, shares this feature (Rogers et al. 1986).

c-Jun and c-Fos are both degraded by the 26S proteasome complex (Treier et al. 1994; Papavassiliou et al. 1992; Ciechanover et al. 1991). However, several aspects of the regulation of AP-1 turnover are controversial, perhaps suggesting the participation of multiple pathways. Treier *et al.* showed that c-Jun is ubiquitinated and degraded by the 26S proteasome pathway *in vivo* (Treier et al. 1994) and in addition that the δ domain, a 27 amino acid sequence located at NH₂-terminus of the protein, is essential for c-Jun ubiquitination and its subsequent proteolysis. In line with these findings, the oncogenic counterpart v-Jun, which lacks the δ domain due to an in frame-deletion, was shown to have elevated protein stability in comparison to c-Jun (Treier et al. 1994). JNK

binds to the δ domain of c-Jun and consequently targets its substrate for ubiquitinylation (Fuchs et al. 1996). In contrast, *in vitro* studies of AP-1 ubiquitinylation using reconstituted enzyme systems showed that c-Jun lacking the δ domain and c-Fos lacking the PEST motif, a common sequence involved in the rapid destruction of short-lived proteins (Rogers et al. 1986), can be efficiently ubiquitinated (Hermida-Matsumoto et al. 1996). It was also reported that degradation of c-Jun by the 26S proteasome pathway does not require ubiquitinylation of the protein *in vitro* (Jariel-Encontre et al. 1995). Another number of substrates, including ornithine decarboxylase, is degraded by the 26S proteasome in a ubiquitin-independent manner. c-Jun might contain intrinsic signals for ubiquitin-independent recognition by the 26S proteasome complex and subsequent degradation. In addition, a role for calpains (calcium-dependent cysteine proteases) in the degradation of c-Jun and c-Fos has been shown. However, these proteases may have only limited involvement in AP-1 turnover, as they are restricted to the cytoplasm (Jariel-Encontre et al. 1995).

Taken together, these data imply that the regulation of c-Jun and c-Fos turnover is complex and controversial but also establish that the major proteolytic pathway for their regulation involves the 26S proteasome pathway. Whether ubiquitinylation is an absolute requirement for the proteolysis *in vivo* of these proteins, and whether different pathways co-exist, possibly depending on the physiological conditions of the cell or the cell type, are still open issues.

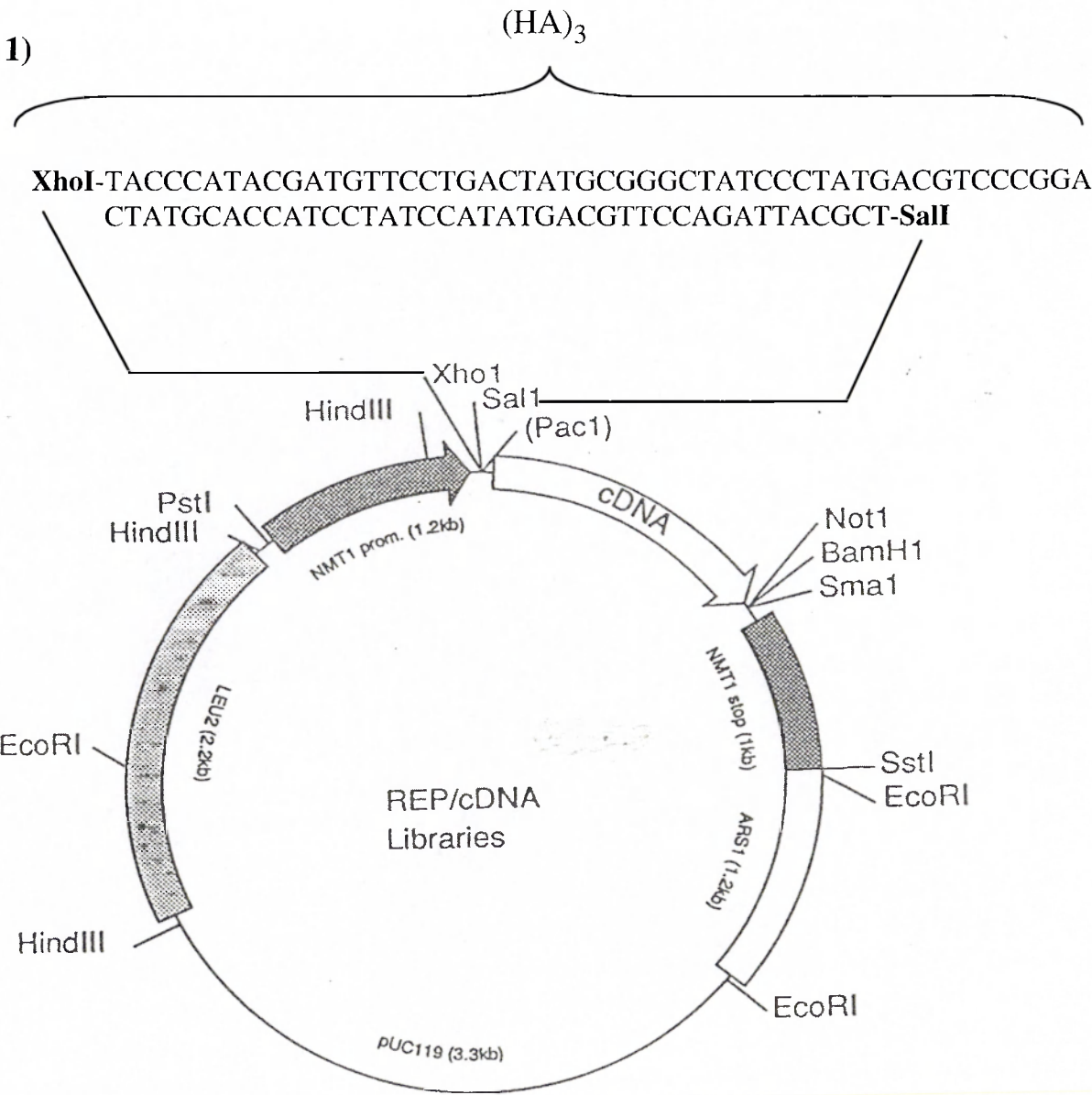
One interesting general observation that has recently come to light is the inverse correlation between the stability of transcriptional activators and their activation domain potency *in vivo* (Molinari et al. 1999). It seems that the rate of degradation of transcription factors by the 26S proteasome pathway strongly depends on the degree of

their activity, as highly potent transcription activators are processed very rapidly in mammalian cells. In order to be degraded these activators must interact with their targets.

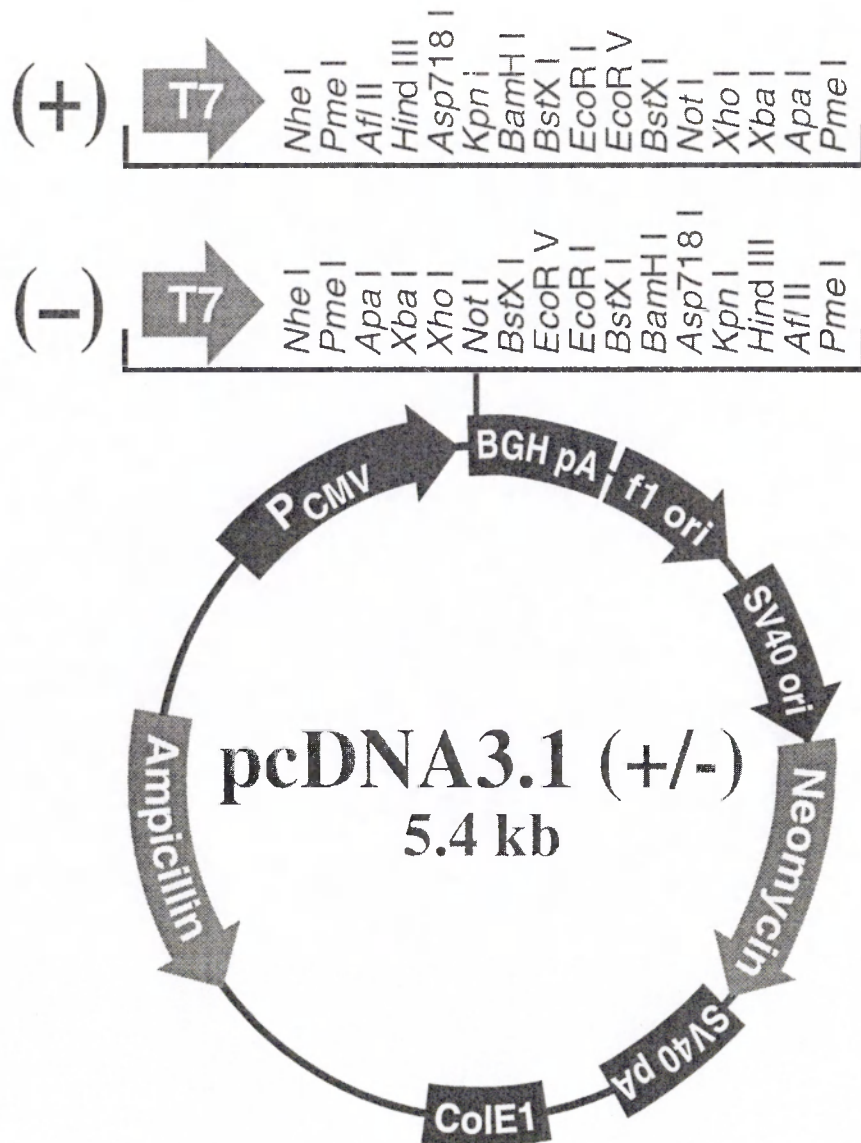
AP-1 and drug resistance

Some studies have suggested a role for AP-1 factors in the modulation of drug resistance in cancer cell lines. Multi-drug resistance MCF-7 breast cancer cells exhibited elevated expression of *c-jun* and *c-fos* genes together with elevated transcriptional and DNA binding activities of AP-1 (Daschner et al. 1999). AP-1 modulation of the *MDR-1* gene, which contains a putative AP-1 binding site in its promoter region with consequent increase of MDR-1 mRNA and protein level have also been documented. Furthermore, c-Jun mRNA level and DNA binding activity of AP-1 were also reported to be elevated in etoposide resistant human leukemia cells (Ritke et al. 1994). Other reports have shown a positive correlation between AP-1 activation and glutathione-S-transferase levels in drug resistant cancer cell lines (Moffat et al. 1994; Puchalski and Fahl 1990).

A.4) Vectors maps



2)



440 450 460

CCAGCCTGCGGCCGCTTAATTAAAGTTTAAACGGATCCCCGGG

NotI *PacI* *BamHI*

XhoI (1) blunted by Klenow

linker insertion

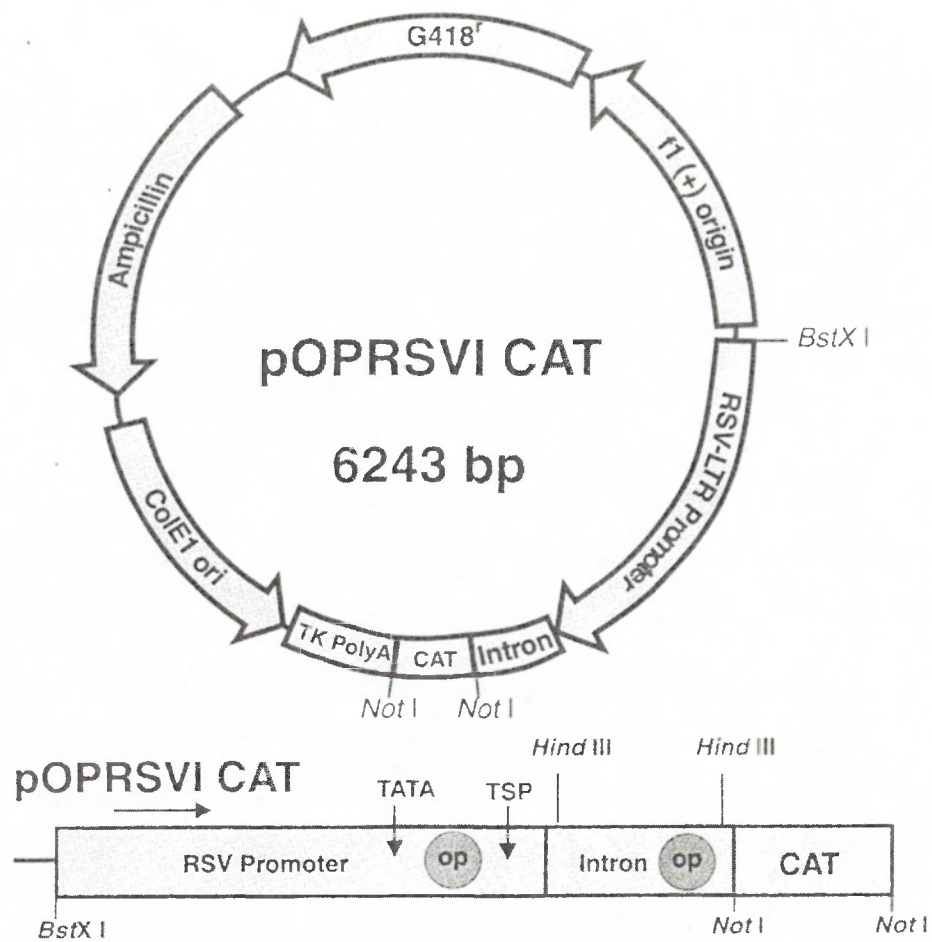
Polylinker sequence

k/o *NotI* *XhoI* *MluI* *HpaI* *ClaI* *NotI*

GGCCTCGAGACGCGTGTTAACATCGATGCGGCCGCATCG
AGCTCTGCGCACAAATTGTAGCTACGCCGGCGTAGCCTAG

1/2 *BamHI*

4)



Polylinker sequence

XhoI

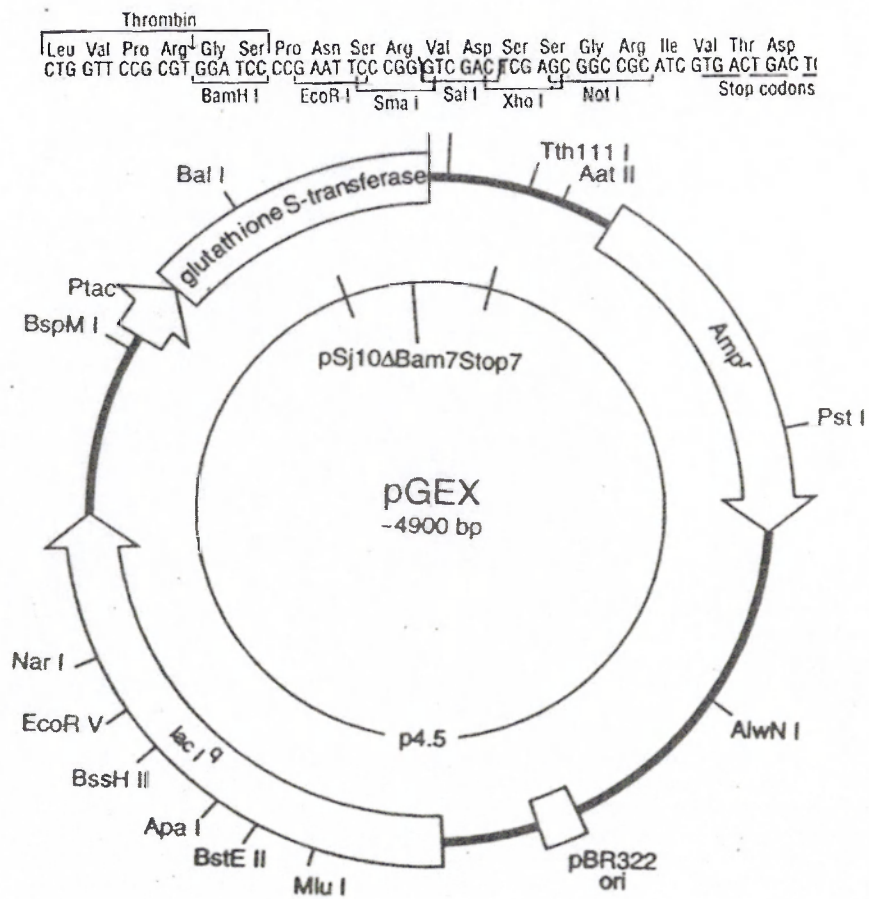
NotI

ClaI

CTCGAGATCGATGCGGCCGC

5)

Polylinker sequence



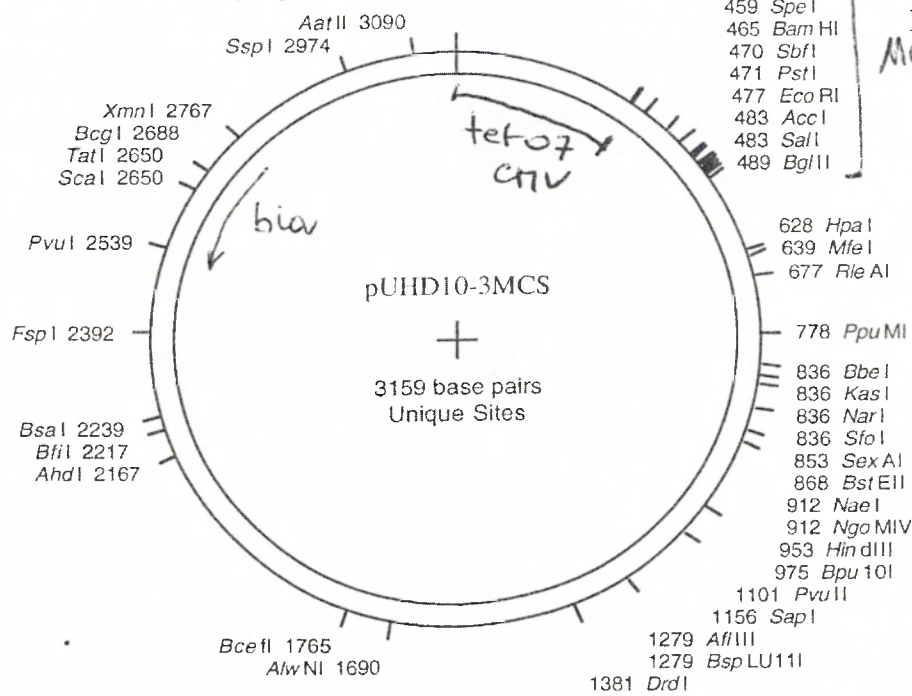
6)

1 *Scl*I
1 *Xho*I

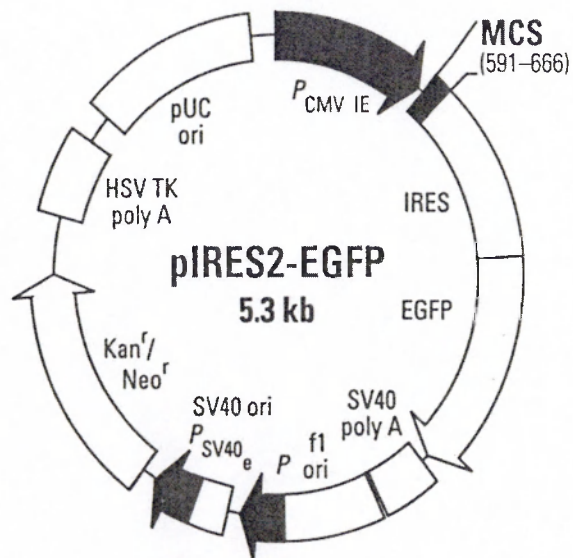
304 *Acc*65I
304 *Bae*I
304 *Kpn*I
308 *Sma*I
308 *Xma*I
336 *Stu*I
382 *Bsm*BI
414 *Bbs*I
439 *Bst*DSI
439 *Mlu*113I
439 *Sac*II
445 *Not*I
446 *Eag*I
459 *Spe*I
465 *Bam*HI
470 *Sbf*I
471 *Pst*I
477 *Eco*RI
483 *Acc*I
483 *Sal*I
489 *Bgl*II

Polylinker sequence

MCS



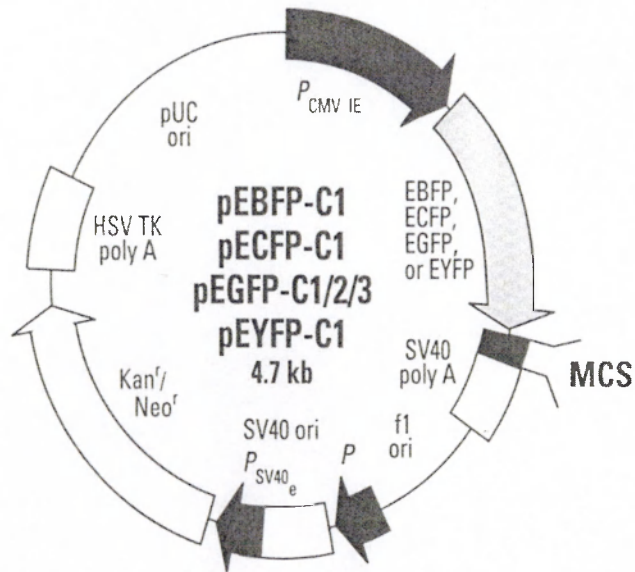
7)



Polylinker sequence

591	601	611	621	631	641	651	661
GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC...IRES							
<i>NheI</i> <i>Eco47 III</i>		<i>BglII</i> <i>XhoI</i>	<i>SacI</i> <i>Ecl136 II</i>	<i>Hind III</i>	<i>EcoRI</i>	<i>PstI</i>	<i>SalI</i> <i>AccI</i> <i>KpnI</i> <i>Asp718 I</i> <i>ApaI</i> <i>Bsp120 I</i> <i>BamHI</i> <i>SacII</i> <i>XmaI</i> <i>SmaI</i>

8)



C1 MCS
EBFP/ECFP/
EGFP/EYFP

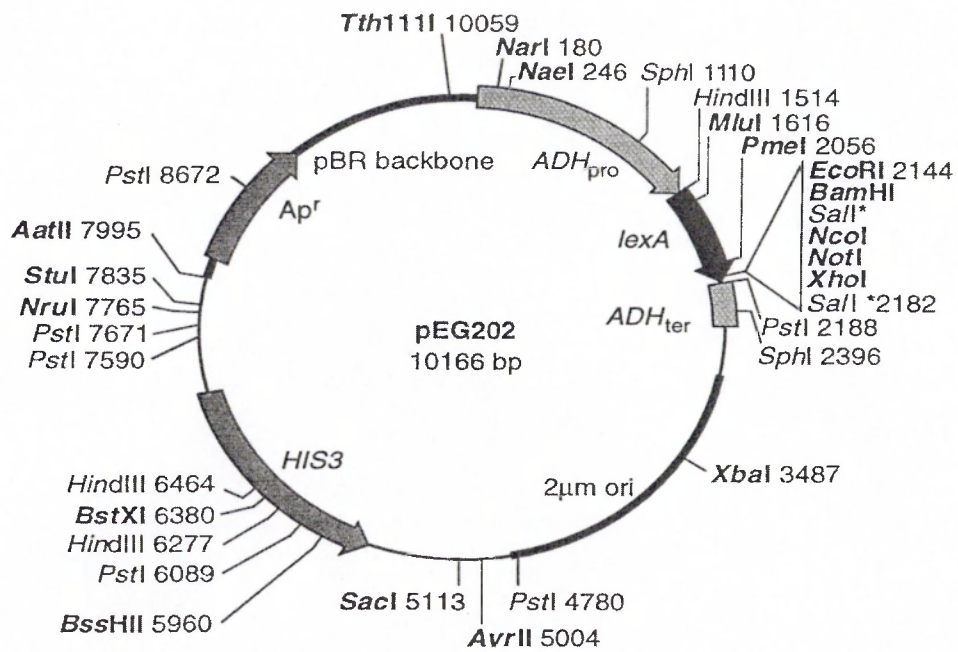
TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC GGA TCT AGA TAA CTG ATC A

*BspE*I⁺ *Bgl*II *Xho*I *Sac*I *Hind*III *EcoR*I *Pst*I *Sal*I *Kpn*I *Apa*I *Ban*HI *Xba*I[∞] *Bcl*I[∞]

*Eco*N36 II *Acc*I *Asp*118 I *Sac*II *Bsp*120 I *Xma*I *Sma*I

CC-MCS

9)



Polylinker sequence

<u>EcoRI</u>	<u>BamHI</u>	<u>SalI*</u>	<u>NcoI</u>	<u>NotI</u>	<u>XhoI</u>	<u>SalI*</u>
GAA TTC CCG GGG ATC CGT CGA CCA TGG CGG CCG CTC GAG TCG AC						